

CELLULAR MECHANISMS FOR  
THE REGULATED DEGRADATION OF ALDOLASE B

By

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Stress-induced degradation of abundant long-lived cytosolic housekeeping proteins was examined using liver aldolase B as a model protein. Heat stress increases ubiquitination that mediates autophagic degradation of long-lived proteins in E36 Chinese hamster cells. During starvation, major multiubiquitinated proteins (*e.g.*, Ub68) increased in lysosomes (rat liver and Fao hepatoma cells) and were antigenically characterized as aldolase B-ubiquitin conjugates. Compared with controls, heat stress increased endogenous aldolase A activity in lysosomes of E36 cells by >twofold. Heat stress was non-permissive for ubiquitination in E36-derived ts20 mutant cells and failed to increase aldolase activity in ts20 lysosomes. Myc-tagged aldolase B (RABM) expressed in E36 cells underwent limited proteolytic processing in lysosomes that failed

to occur in heat stressed ts20 cells. The results suggested that during stress (starvation or heat), aldolase A and aldolase B can undergo ubiquitin-mediated autophagic degradation. Long-lived protein degradation was a continuous function of temperature, indicating heat stress-induced rates were due to thermodynamic stimulation of chemical reactivity. Lysosomal inhibitors distinguished proteolysis in lysosomes from that in cytosol. Complete autophagic degradation to amino acids in lysosomes was highly temperature-dependent compared to a relatively constant rate in cytosol. HA-tagged human aldolase B (HAHAB) in Fao cells and RABM in E36 cells underwent proteolysis in cytosol that had temperature-dependence paralleling complete degradation of proteins in lysosomes. Lysosomal degradation was ubiquitin-dependent (blocked in heat stress ts20 cells), but cytosolic proteolysis of RABM was not. Results suggest a possibly shared temperature-dependent cytosolic mechanism that limits rates for partial cytosolic proteolysis and complete lysosomal degradation of long-lived proteins. Three peptide motifs for signal-mediated targeting to lysosomes during starvation occur in aldolase B. These were mutated in RABM. Starvation-induced degradation of mutant and wildtype RABM expressed in HuH7 human hepatoma cells were measured. Starvation-induced degradation of RABM (aldolase B) specifically required a glutamine at residue #111 suggesting that the corresponding peptide motif, IKLDQ, is a targeting signal functionally demonstrated in living cells. Evidence was provided for three previously unknown mechanisms for stress-regulated degradation of aldolase B: (1) ubiquitin-

mediated autophagic degradation in lysosomes, (2) temperature-dependent cytosolic proteolysis during heat stress, and (3) signal-mediated degradation during starvation.

## CHAPTER 1: INTRODUCTION

### General Concepts of Protein Degradation

In cells, different proteins have different functions and occur at different levels as needed. The functional activities and locations of proteins are regulated to integrate with each other, maximizing survival. Proteins can be regulated by a variety of mechanisms, but available concentration of each protein fundamentally determines maximal function (Doherty and Mayer, 1992). Cells adapt to environmental change by altering amounts of different proteins. Some increase, others decrease, and the rest are constant (Doherty and Mayer, 1992). Such adaptation of different proteins requires preferential mechanisms for regulating synthetic or degradative rates in response to environmental change.

Under constant conditions, protein synthesis is independent (zero order relationship) of the protein concentration, but degradation is directly proportional (first order relationship) to protein concentration (Doherty and Mayer, 1992). Synthesis increases protein concentration, causing degradation to increase until synthetic and degradative rates are equal. In this way, a balance between synthesis and degradation determines the available concentration of a protein. If environmental conditions change, then cells can adapt protein concentrations by modulating synthesis or degradation

(Olson, et al., 1992; Mortimore, 1987). This study examines mechanisms of protein degradation that respond to environmental changes.

Continual synthesis and degradation results in constant turnover of proteins which can be described by either the fractional degradation rate or the half-life of the protein. The fractional degradation rate of a protein (degradative rate constant),  $k_d$ , is defined as the fraction of the initial protein degraded in a given time. The  $k_d$  is calculated from measurements of labeled protein lost per time. Half-life,  $t_{1/2}$ , is defined as the time for turnover of half the protein. Under equilibrium conditions,  $k_d$  and  $t_{1/2}$  are constant and directly related to each other by  $t_{1/2} = \ln(2)/k_d$ , allowing calculation of  $t_{1/2}$  from experimentally determined  $k_d$  (Doherty and Mayer, 1992).

Proteins are categorized as short-lived for  $t_{1/2} < 1$  h or long-lived for  $t_{1/2} > 1$  h. For short-lived proteins, protein concentrations respond more to changes in synthesis (Olsen, et al., 1992). For long-lived proteins, protein concentrations respond more to changes in degradation (Olsen, et al., 1992; Mortimore and Poso, 1987). Detailed reasoning for this is described elsewhere (Doherty and Mayer, 1992). Many short-lived proteins utilize a well-characterized mechanism for degradation by a cytosolic protease complex called the proteasome (Rock, et al., 1994; Ciechanover and Schwartz, 1994; Hochstrasser, 1992). Relative to short-lived proteins, mechanisms for the degradation of long-lived proteins are poorly characterized. This dissertation examines mechanisms for stress-inducible degradation of a long-lived cytosolic enzyme, fructose 1,6-diphosphate aldolase (see next section).

Mechanisms for degradation of proteins become enhanced during environmental stress. Increased temperature (Bates, et al., 1982; Hough and Reichsteiner, 1984) or nutrient deprivation (Mortimore and Poso, 1987) are known to increase the degradation of long-lived proteins. Stress-induced degradative mechanisms are of special interest, because they mediate regulated changes and themselves must be regulated (Mortimore, et al., 1987; Mortimore and Poso, 1987; Olson, et al., 1990). Any mechanism that can be triggered by environmental stress lends itself to experimental manipulation. Such a mechanism can be modulated simply by changing experimental conditions. Furthermore, mechanisms required for a stress-induced degradation can be shown to be specific by lack of effect on basal mechanisms. For example, cells respond to starvation by increasing the degradation of long-lived proteins. 3-Methyladenine is a drug that specifically blocks the enhanced degradation without affecting basal degradation. 3-Methyladenine is a potent inhibitor of autophagy, a mechanism for delivering proteins to lysosomes for degradation. Such results provide evidence that autophagy plays a role in enhanced degradation but not basal degradation of proteins.

#### Background for Fructose 1,6-Diphosphate Aldolase B

In the next section, I discuss potential mechanisms for the degradation of the liver isoform of fructose 1,6-diphosphate (FDP) aldolase, called aldolase B. Molecular mechanisms for the degradation of aldolase B have not been examined, but preliminary examination indicated potential roles for ubiquitination and signal-mediated delivery to lysosomes. Before examining the degradation of aldolase B, this section serves to

familiarize the reader with aldolase B in relation to other aldolase isoforms. There are two major classes (I and II) of FDP aldolase that have no sequence homology (Alefounder et al., 1989) and utilize different catalytic mechanisms (Rutter et al., 1966). Class II aldolases are only found in microorganisms and are considered no further here, but class I aldolases are represented in all taxons (Rutter et al., 1966). Microorganisms variably lack class I aldolase. For example, some strains of *E.coli* contain class I aldolase (Alefounder et al., 1989), whereas strain JM83 lacks class I activity (Sakakibara et al., 1989). Protozoa and multicellular eukaryotes contain class I aldolases.

The FDP aldolase isoforms of mammals are the best characterized. Most studies of FDP aldolase degradation examine mammalian isoforms. Mammalian aldolase isoforms are synthesized from three separately regulated genes coding different proteins. Muscle, liver, and brain each express one predominant isoform designated A, B, and C, respectively (Rutter et al., 1966). As such, liver aldolase and aldolase B are synonyms. Likewise, aldolase A is also called muscle aldolase, and aldolase C is referred to as brain aldolase. Most tissues, including embryonic, contain combinations of aldolase A and C, but aldolase B appears to be exclusively expressed in liver and kidney cells (Rutter et al., 1966).

Aldolase classes and isoforms are distinguished by clear differences in their enzymatic properties. For example, class I aldolase like that in mammals is totally functional in the presence of EDTA, but class II enzymes of *E. coli* and other microbes are completely inhibited. The three mammalian isoforms (A, B, and C) can be

distinguished by differences in specific activity, sensitivity to carboxypeptidase A, and kinetics ( $V_{\max}$  and  $K_m$ ) for different substrates (Rutter et al., 1966).

Aldolases A, B, and C also are characterized by distinct native epitopes. Thus, antibody to one poorly recognizes the others. However, an antibody against a specific isoform for one animal will similarly recognize the same isoform of a different species, at least amongst mammals (Penhoet and Rutter, 1975). Native and denatured epitopes of aldolase B have also been shown to be distinct from each other (Reznick et al., 1985). Chemical denaturation of aldolase B before immunization resulted in antibody that failed to immunoprecipitate native enzyme activity but could precipitate degradative fragments of aldolase B. Since aldolase has stable structure that spontaneously refolds into native conformations, only fragments sufficiently denatured by degradation were detected by antibody against the denatured aldolase B (Reznick et al., 1985). Anti-native aldolase B had converse immunoreactivity. Thus, it was proposed that three-dimensional conformation is important for antibody recognition of native surface epitopes, whereas the native structure buries and masks denatured epitopes (Reznick et al., 1985).

The three dimensional structure of all class I aldolase isoforms is conserved from bacteria to humans (Alefounder, et al., 1989). Secondary and tertiary structures of aldolase are very stable. This is true at the quaternary level, too. Aldolase occurs as very stable tetramers that do not undergo subunit exchange after synthesis (Lebherz, 1975; Lebherz, 1972). Different aldolase isoforms co-synthesized in the same cell randomly associate into stable heterotetramers. Thus, immunoprecipitation with

antibody against one isoform specifically precipitates antigenically unrelated isoforms in the same tetramer. However, surface charge and pI on different isoforms varies. Thus, isozymes containing different ratios of two isoforms (e.g. A<sub>4</sub>, A<sub>3</sub>B, A<sub>2</sub>B<sub>2</sub>, AB<sub>3</sub>, or B<sub>4</sub>) can be separated by isoelectric focusing (Lebherz, 1972; Penhoet and Rutter, 1975). Comparison of X-ray crystallographic results shows that secondary and tertiary structure between muscle, liver, and *Drosophila* aldolases are very close (Berthiaume et al., 1993).

Aldolase isoforms vary in their capacity to bind actin cytoskeletons. In the only paper to measure cytoskeletal association of all three isoforms (A, B, and C), investigators claimed that different purified isoforms of aldolase had tissue-specific affinity for cytoskeletal preparations isolated from different tissues (Kusakabe et al., 1997). After mixing crude cytoskeletons with a known amount of purified aldolase, they pelleted the mixture and then only measured unbound activities in supernatants. They failed to show removal of endogenous aldolase from cytoskeletal preparations, so measurements could be contaminated and include competitive effects. However, their results were consistent with other investigators in that relative tightness of binding to actin cytoskeleton is greatest to least, aldolase A, aldolase B, then aldolase C (Clarke, et al., 1982; O'Reilly and Clarke, 1993).

Four "isotype specific" sequences contain most of the variation and the carboxyl terminus has the greatest diversity (Marchand et al., 1988; Paoletta et al., 1984; Rottmann et al., 1984). The carboxyl terminus is important in determining isoform-specific catalytic properties (Berthiaume et al., 1993; Gamblin et al., 1991; Penhoet and

Rutter, 1975). Aldolase B has the lowest specific activity amongst aldolase isoforms and is least sensitive to proteolytic alterations in this region. It can lose up to four C-terminal residues without affect its enzymatic activity (Berthiaume et al., 1993; Horecker et al., 1985). Even with 10 to 20 residues removed by carboxypeptidase, aldolase B retains almost half its activity (Penhoet and Rutter, 1975). However, aldolase A absolutely requires a carboxyl terminal tyrosine at residue 364 (Y364) for activity that is about 20 times greater than aldolase B, and when aldolase A loses its C-terminus the remaining activity resembles that of aldolase B (Takahashi et al., 1989; Gamblin et al., 1991). These results indicate that alterations in the carboxyl terminus of aldolase B (such as epitope tagging) are less likely to affect its properties than other aldolase isoforms.

All fructose 1,6-diphosphate aldolase enzymes catalyze a reversible reaction essential for glycolysis and gluconeogenesis. Aldolase B is the liver form of this enzyme expressed to the exclusion of other forms of aldolase in normal hepatocytes (Asaka, et al., 1983). Since liver is the only organ known to export glucose (Stein and Arias, 1976; Stryer, 1988), aldolase B performs gluconeogenesis for the entire body. Liver also provides amino acids during starvation and in three days can lose nearly half its weight (and protein content), a faster loss than other tissues (Wing et al., 1991). In this regard, aldolase B is an example of an abundant cytosolic protein that undergoes enhanced degradation during starvation which yields amino acids for export to other organs. Liver amino acids can also be converted to glucose or ketone bodies to provide energy sources during starvation. Abundant long-lived liver enzymes that mediate

glycolysis/gluconeogenesis, like aldolase B and glyceraldehyde phosphate dehydrogenase (GAPDH) are poised between two mutually exclusive functions: catalyzing carbohydrate metabolism and providing amino acids for protein biosynthesis or energy metabolism.

Liver and kidney are the only tissues having predominant aldolase B expression (Penhoet and Rutter, 1975). Both organs demonstrate enhanced degradation of proteins during amino acid starvation (Olsen, et al. 1990). Liver and kidney also receive the largest fraction of the body's basal blood flow, 27% and 22%, respectively, followed by 15% for muscle and 14% for brain (Guyton, 1979). This is consistent with involvement of the two former organs in regulating serum components and contribution of aldolase B to serum glucose and amino acids during starvation. Among aldolase isoforms, aldolase B contributes a greater role in carbohydrate and protein metabolism that is not limited to local cells and tissues, but extends to the entire body.

### Mechanisms for Degradation Aldolase B

#### Inactivation by Limited Proteolysis

Alteration of aldolase A and B's carboxyl termini was proposed to down-regulate activity (Pontremoli et al., 1982; Pontremoli et al., 1979). During starvation, aldolase B activity is lost from liver faster than loss of immunoreactivity. Thus, investigators suggested that starvation-induced inactivations precede total degradation of aldolase A and B, providing more rapid down-regulation of activity (Pontremoli et al., 1979).

Inactivation happens by limited C-terminal cleavage that can maintain native

immunoreactivity and barely affect mobility on SDS-PAGE. One group of investigators proposed phosphorylation near the C-terminus of aldolase as an inactivating mechanism, but this was only demonstrated *in vitro* (Sygusch et al., 1990). More likely inactivation occurs by limited proteolysis which would have a much more profound impact on aldolase A activity than on aldolase B activity (previous section, last paragraph).

The best characterized mechanism for aldolase inactivation is limited proteolysis by a dipeptidyl (two residues per cleavage) carboxypeptidase on lysosomes (Pontremoli and Melloni, 1986; Horecker et al., 1985). The peptidase, cathepsin M, was defined as a cathepsin B or L-like activity associated with the cytosolic surface of lysosomal membranes. During starvation, a lysosomal matrix cathepsin B/L associates with lysosomal membranes, acquires activity at neutral pH, and becomes exposed to the cytosolic compartment as cathepsin M (Pontremoli et al., 1984; Pontremoli et al., 1982). Specific cleavage sites have been characterized *in vitro* (Horecker et al., 1985).

Starvation-induced *in vivo* loss of liver aldolase specific activity correlated with loss of carboxyl terminal tyrosine residues which was estimated by isolating aldolase B and measuring lost tyrosine content in an acid soluble peptide released from the C-terminus with subtilisin (Pontremoli et al., 1982). According to such experiments, inactivated aldolase B constitutes about 40% of the aldolase in liver after 60 hours of starvation. Most of the inactivated aldolase B must occur in cytosol, because only a small fraction of total aldolase (about 10%) is associated with pelletable fractions from liver (Kominami et al., 1983; Kopitz et al., 1990). Moreover, intralysosomal

degradation of aldolase is rapid (see below), precluding accumulation of an inactivated form in such organelles. The results are consistent with inactivation occurring in the cytosolic compartment, albeit by an activity associated with the cytosolic surface of lysosomes.

#### *In Vitro* Denaturation of Aldolase and Need for *In Vivo* Mechanism

Except for 20 “loose” amino acid residues at the carboxyl terminus, the stability of aldolase structure resists proteolysis and requires denaturation for rapid *in vitro* proteolysis to proceed. In optimized conditions with cathepsin D, only about 20 amino acids of aldolase A can be digested from its carboxyl terminus (Offermann et al., 1983). *In vitro* proteolysis with either meprin (a metalloproteinase) or a mixture of lysosomal proteases produces only a slight increase in SDS-PAGE mobility, and the remaining part of aldolase A has a thermal stability identical to the native enzyme (Bond and Offermann, 1981). Purified aldolase B digested with a lysosomal extract also only undergoes limited proteolysis, losing some but not all its activity (Chappel et al., 1978). However, denaturing pretreatment with disulfides like glutathione (Offermann et al., 1983) or cystine (Bond and Offermann, 1981) permits extensive proteolysis to occur. Given this, there must be a “denaturing” mechanism *in vivo* to allow degradative turnover of aldolase to occur. Interestingly, aldolase B sequestered *in vivo* and isolated with lysosomes is susceptible to more extensive *in vitro* proteolysis in the lysosomal preparations (Kominami, et al., 1983; Ueno and Kominami, 1991). Apparently,

aldolase B becomes sensitized to proteolysis by a mechanism in cytosol before sequestration or in intact lysosomes after sequestration.

After loading aldolase A into endosomes at 19°C, temperature can be raised to 37°C allowing rapid fusion of endosomes with lysosomes. Thus, intralysosomal degradation can be measured. By this method, native or variously denatured and inactivated aldolases all degrade rapidly with similar rates ( $t_{1/2} < 10$  min). Since its  $t_{1/2}$  is normally many hours in cytosol, sequestration appears rate limiting for lysosomal degradation of aldolase (Bond and Aronson, 1983). The results of the endocytic loading experiments indicate that a mechanism for denaturing and sensitizing aldolase to proteolytic attack can occur in lysosomes or other organelles of the endosomal pathway. Thus, a cytosolic denaturing mechanism is not necessary for intralysosomal degradation of aldolase, but a role in delivery of aldolase to lysosomes cannot be excluded.

The tetrameric structure of aldolase is well established (Leberherz, 1972). This quaternary structure seems important for aldolase stability. Recently, Beernink and Tolan have identified specific amino acids that mediate subunit interaction between aldolase monomers (Beernink and Tolan, 1996). Significantly, a mutant with only two amino acid changes retains enzymatic activity but exists as monomers. These monomers (and dimers created with single amino acid mutations) are more sensitive to chemical or thermal inactivation, indicating "looser" structure. Thus, tetrameric association improves structural stability.

Lysosomes are acidic inside (pH ~5), and reversible *in vitro* dissociation of aldolase into monomers occurs at pH < 6.0 (Beernink and Tolan, 1996). Acidic pH affecting aldolase structure is also indicated by reduced enzymatic activity. Thus, intralysosomal pH would have a denaturing effect that could permit lysosomal proteolysis. However, other investigators incubated aldolase B with crude lysosomal hydrolases at acidic pH and failed to get significant proteolysis (Chappel et al., 1978). Apparently, low pH is insufficient to permit further proteolytic attack, and aldolase denaturation must require other factors. Consistent with this, lysosomes purified from liver contain detectable aldolase B which is susceptible to proteolysis when the intact lysosomes are incubated *in vitro* at pH < 5.5 (Kominami, et al., 1983; Ueno and Kominami, 1991). The endocytic loading experiments described above indicate lysosomes (or an endocytic compartment) must contain denaturing factors, but this does not exclude the possibility of a cytosolic denaturation of aldolase B before delivery to lysosomes.

Aldolase A has been radiolabeled, inactivated and denatured, then microinjected into cultured cells (Hopgood et al., 1988; Knowles et al., 1989). The procedure delivers the enzyme into cytosol where it normally resides. As with endocytic loading, degradation rates for aldolase were similar whether the enzyme was native, inactivated, or denatured. Denaturation of aldolase is not rate limiting for degradative steps before lysosomes as well as within them. Degradation of aldolase microinjected into cytosol matched expected turnover for aldolase ( $t_{1/2} \cong 30$  hours) which was much slower than for

aldolase loaded into lysosomes ( $t_{1/2} < 10$  minutes). Assuming that degradation occurs within lysosomes, this suggests that sequestration of aldolase is rate-limiting for its turnover (Bond and Aronson, 1983; Bond and Offermann, 1981; Hopgood et al., 1988; Knowles et al., 1989).

Though *in vitro* studies indicate denaturation of aldolase structure is necessary for proteolysis, *in vivo* denaturation is not rate-limiting for delivery to or degradation within lysosomes. These data support a model in which aldolase delivery to lysosomes is rate limiting followed by rapid intralysosomal proteolysis which would need a faster denaturing mechanism. Lysosomal acidity might facilitate denaturation of aldolase, but acidity alone is insufficient for sensitizing stable aldolase structure to attack by acid hydrolases. The above data do not exclude a cytosolic denaturing mechanism for aldolase, but indicate that such a mechanism is not rate limiting and not necessary for intralysosomal proteolysis. The next two sections review mechanisms for the delivery of cytosolic proteins to the lysosomal lumen, a process that appears rate-limiting for aldolase degradation.

### Autophagy

Autophagy is the sequestration of cytoplasm into vesicles for intralysosomal degradation and is the only mechanism proposed for the complete degradative turnover of aldolase. There are two forms of autophagy: macroautophagy and microautophagy. Commonly, investigators use the term "autophagy" to mean macroautophagy which is

the better characterized form. Likewise, "autophagy" used here refers to macroautophagy, and reference to "microautophagy" will be explicit.

Autophagy (macroautophagy) begins with a ribosome-free portion of endoplasmic reticulum engulfing a portion of cytoplasm. Autophagy non-selectively sequesters cytosol and organelles into distinct autophagic vacuoles. The autophagic vacuoles mature including a process of acidification. Finally, mature autophagic vacuoles fuse with lysosomes producing autolysosomes in which degradation occurs (Dunn, 1990; Dunn, 1990). Enhanced autophagy is initiated by amino acid starvation and is also regulated by hormones (Hendil et al., 1990; Seglen and Bohley, 1992). In the model of Figure 1-1, non-selective autophagy is represented by the upper pathway in the diagram. The lower pathway of Figure 1-1 (Receptor-Mediated Targeting) is discussed in the next section.

Microautophagy seems simpler than macroautophagy. During microautophagy the lysosomal membrane itself invaginates, extending a finger of cytosol into the lysosome. This protrusion pinches off producing an intralysosomal vesicle that gets degraded with its cytosolic content. Apparently, microautophagy can occur *in vitro*, but the complexity of macroautophagy has not been reconstituted (Seglen and Bohley, 1992). Unlike macroautophagy which has discrete autophagic vacuoles, microautophagy fails to produce separable organellar compartments. Thus, microautophagy requires time-consuming electron microscopy to demonstrate its existence and remains poorly characterized (Seglen and Bohley, 1992).

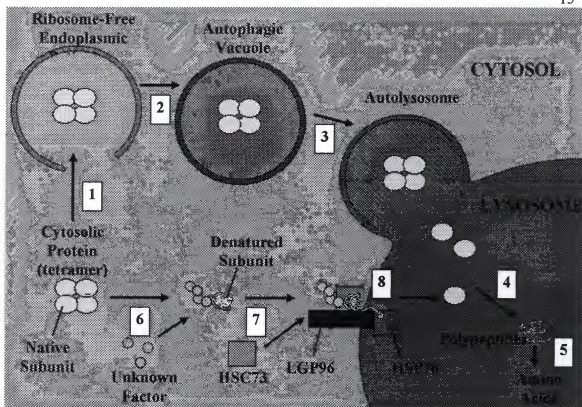


**Figure 1-1: Mechanisms for Stress-Induced Degradation of Cytosolic Proteins in Lysosomes.** Autophagy (upper pathway) and receptor-mediated targeting (lower pathway) were proposed for stress-induced delivery of cytosolic proteins to lysosomes for degradation; the arbitrary cytosolic protein is shown as a tetramer (aldolase B occurs as a tetramer); components of the pathways are labeled on the diagram; processes are labeled by boxed numbers: 1, association with or engulfment by autophagic membranes; 2, sequestration into double-membrane bound autophagic vacuole; 3, maturation of autophagic vacuole (acidification and acquisition of lysosomal hydrolases); 4, proteolysis into polypeptide fragments; 5, complete degradation to amino acids; 6, disassembly and denaturation of structure by an unknown factor; 7, association with a receptor complex on the lysosomal surface; 8, translocation across the lysosomal membrane.

Selective mechanisms of autophagy exist. Methylotrophic yeast use a selective mechanism of autophagy to degrade peroxisomes when switched from methanol to a different carbon source, and electron microscopic morphology shows a mechanism

topologically identical to microautophagy (Tuttle et al., 1993). Occurrence of selective microautophagy in higher organisms has not been demonstrated, and a role for microautophagy in degradation of aldolase has not been studied. If selective autophagy does occur for aldolase, then a receptor-mediated complex would be required for selectivity. Such a receptor complex could form on the lysosomal surface (Fig. 1-1, lower pathway), followed by microautophagic sequestration. However, receptor function does not distinguish microautophagy and macroautophagy, so Figure 1-1 only distinguishes non-selective autophagy (upper pathway) from a hypothetical selective process that might include microautophagy (lower pathway).

Autophagy (macroautophagy) is a subject of active research, producing almost 300 related papers in just the last five years. FDP aldolases are generally abundant, commonly known, cytosolic enzyme, and the muscle isoform, aldolase A, is commercially available. Aldolase A and aldolase B have been used as markers for autophagic uptake of cytosol into lysosomes, and degradation of aldolase by autophagy is well established (Henell et al., 1987; Kominami et al., 1983; Kopitz et al., 1990; Seglen and Gordon, 1982; Ueno et al., 1990). Per O. Seglen's laboratory briefly treated starved hepatocytes with cycloheximide to prevent new protein synthesis and estimated degradation rates by loss of enzyme activity. Incubations were short to avoid depletion of autophagic factors (continual autophagy requires new protein synthesis). A lysosomal inhibitor (leupeptin) was used to estimate how much degradation occurred in lysosomes. In this way, starvation-induced degradation of aldolase B was lysosomal occurring at

3.6±0.1 %/h. Other cytosolic enzymes with widely different half-lives were similarly tested. As expected, they had very different total degradation rates. However, lysosomal degradation (3.3-5.3 %/h) and rates of accumulation in organelles during lysosomal inhibition (3.1-3.7 %/h) were similar for all the enzymes. These rates match rates of starvation-induced autophagy (3-5 %/h) and were blocked with 3-methyladenine a specific inhibitor of autophagy. Thus, Seglen concluded that cytosolic enzymes, including aldolase B, are degraded via non-specific autophagy (Kopitz et al., 1990). Interestingly, of all the enzymes tested by Seglen, only two were exclusively degraded in lysosomes, aldolase B and lactate dehydrogenase H (Kopitz et al., 1990). Coincidentally, both aldolase B and lactate dehydrogenase H contain sequence motifs for receptor-mediated targeting to lysosomes for degradation.

#### Receptor Mediated Targeting to Lysosomes

A pentapeptide sequence (KFERQ) of RNase A was shown to mediate its delivery to lysosomes for degradation during nutrient deprivation (Dice and Chiang, 1988). Characterization of this signal identified a motif contained in a subset of cellular proteins that undergo enhanced degradation in lysosomes during nutrient deprivation (Wing et al., 1991). The motif has been proposed as a binding site for a molecular chaperone called HSC73 which then delivers motif-containing proteins into lysosomes in an ATP-dependent manner (Chiang et al., 1989). The mechanism (Figure 1-1, lower pathway) also requires intralysosomal HSP70, and a recently identified lysosomal membrane receptor, LGP96 (Cuervo, et al. 1996). J. Fred Dice proposed that this

pathway occurs by a mechanism analogous to transmembrane transport of proteins during organellar biogenesis (Dice and Chiang, 1988; Terlecky et al., 1992; Wing et al., 1991).

Recently, Dice's group made a major advance by identifying a receptor protein in the target membrane of lysosomes that mediated transmembrane translocation (Cuervo and Dice, 1996). The lysosomal membrane protein, LGP96, was demonstrated to be a rate limiting component in this degradative pathway. CHO cells overexpressing human LGP96 by two to three fold had correspondingly increased degradation of long-lived proteins. Furthermore, lysosomes isolated from these cells had two to three fold higher ATP-dependent uptake of the glycolytic enzyme GAPDH (glyceraldehyde 3-phosphate dehydrogenase), a known substrate for this pathway (Cuervo and Dice, 1996). Unfortunately, GAPDH does not contain sequence matching previously established criteria for the receptor-mediated targeting motif. The previous criteria define necessity for an "essential" glutamine, but in GAPDH, asparagine apparently can substitute for glutamine (Dice, personal communication). As just described, receptor function has been demonstrated in living cells; however, evidence for *in vivo* function of the signal peptide is lacking.

A recent study found that the conformation of signal motifs were inappropriate to mediate the receptor-mediated lysosomal targeting pathway (Gorinsky et al., 1996).

Some proteins known to contain motifs for the pathway, including RNase A, also have known three dimensional structures. The peptide signal motifs are supposed to be

recognized by cytosolic HSC73 which is required for delivery to lysosomes. However, signals on proteins of known structure are either embedded or occurred in  $\alpha$ -helical conformations. Since hsp70-type chaperones require extended conformations for recognition, the investigators concluded that HSC73 would require other unknown factors to relax a substrate protein's structure and allow signal-mediated targeting to lysosomes to occur (Gorinsky et al., 1996). The lower pathway in Figure 1-1 summarizes receptor-mediated targeting to lysosomes, including an unknown factor that alters the structure of the cytosolic protein.

Except for the work presented in this dissertation, no studies have examined any aldolase isoform as a substrate for the receptor-mediated pathway. Aldolase A binds very well to GAPDH presumably for greater glycolytic efficiency (Verlessy and Vas, 1992). Both these proteins appear to be regulated at similar high concentrations in cells (Verlessy and Vas, 1992), and aldolase B and GAPDH undergo similar starvation-induced degradation in cultured cells (Kopitz, et al., 1990). Does aldolase B follow receptor-mediated targeting to lysosomes like GAPDH?

All vertebrate aldolases contain a conserved motif (Fig. 1-2, residues 12-16) for receptor-mediated targeting of cytosolic proteins into lysosomes (Dice and Chiang, 1988; Zhang et al., 1995). In mammalian aldolase B, two additional sequences for the lysosomal targeting motif were found (Fig. 1-2, residues 58-62 and 107-111). Whether any of these three motifs are functional was unknown. Though the lysosomal targeting motif has rather broad criteria (Dice and Chiang, 1988), an aldolase-sized (40 kD)

MAHRFPALT **S**EQKKELSEI AQRIVANGKGILAADESVGTMGNR  
 MAHRFPALT **Q**EQKKELSEI AQRIVANGKGILAADESVGTMGNR  
 1

LQRIKVENTEENRR**Q**FRELLFSVDNSISQSIGGVILFHETLYQKDS  
 LQRIKVENTEENRR**Q**FREILFSVDNSISQSIGGVILFHETLYQKDS  
 44

QGKLFNRILKEKGIVV**G**IKLDQGGAPLAGTNKETTIQGLDGLSER  
 QGKLFNRILKEKGIVV**G**IKLDQGGAPLAGTNKETTIQGLDGLSER  
 90

CAQYKKDGVDFGKWRAVLRI SDQCPSSLAIQENANALARYASIC  
 CAQYKKDGVDFGKWRAVLRIADQCPSSLAIQENANALARYASIC  
 135

QQNGLVPIVEPEVLPDGDHDLHCQYV SEKVLAAYVKALNDHH  
 QQNGLVPIVEPEV I PDGDHDLHCQYVTEKVLAAYVKALNDHH  
 179

VYLEGTLLKPNMLTAGHACTKKYTPEQVAMATVTALHRTVPAA  
 VYLEGTLLKPNMVTAGHACTKKYTPEQVAMATVTALHRTVPAA  
 221

VPSICFLSGGMSEEDATLNLNAI**Y**RCPLRPWKLSFSY**G**RALQAS  
 VPGICFLSGGMSEEDATLNLNAI**N**LCLPKPWKLSFSY**G**KALQAS  
 265

ALAAWGGKAANK**K**ATQEA FMKRAV AN**C**QGYVHTGSSGAAS  
 ALAAWGGKAANK **E**ATQEA FMKRAMAN**C**KGYVHTGSSGAAS  
 300

TQSLFTASYTY  
 TQSLFTACYTY  
 354 364

**Figure 1-2: Amino Acid Sequences of Aldolase B Isoforms Used in This Study.** The upper and lower sequences are for rat and human liver aldolase, respectively. Boldface indicates non-identical residues. Underline indicates pentapeptide signal motifs for receptor-mediated lysosomal targeting. Large arrows point to essential glutamines of the signals as indicated (sequence data from Tsutsumi et al., 1984 and Paoletta et al., 1984).

protein would have only a 7% chance of randomly containing three such signal motifs. In addition to the motifs, aldolase B has properties similar to other substrates of this signal-mediated degradative mechanism: (1) long-lived, (2) cytosolic, (3) housekeeping protein, and (4) degraded in lysosomes by enhanced proteolysis during nutrient withdrawal. Furthermore, aldolase is closely associated with another glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is an established substrate for receptor-mediated targeting to lysosomes (Aniento et al., 1993). Aldolase and GAPDH form a complex that facilitates their sequential roles in glycolysis (Verlessy and Vas, 1992), both are very abundant, and their *in vivo* turnover rates are very similar (Kuehl and Sumsion, 1970), suggesting that they could share degradative mechanisms. Furthermore, the receptor-mediated pathway proceeds by transmembrane translocation into lysosomes by a mechanism like that of organellar biogenesis. Coincidentally, the aldolase isoform of *Trypanosoma brucei* (45% identical to aldolase B) undergoes transmembrane transport during biogenesis of the unique glycolytic organelle of this protozoan (Marchand et al., 1988). Together, these facts suggested that aldolase B was a likely candidate for receptor-mediated targeting to lysosomes via the proposed transmembrane transport mechanism.

#### Ubiquitination and the Degradation of Long-lived Proteins

Ubiquitination is an orderly process whereby a 76-amino acid polypeptide, ubiquitin, is covalently conjugated to other proteins at its carboxyl terminus. In a series of transfers, three enzymes (E1, E2, and E3) covalently bind and pass ubiquitin to the

next protein. E1, called ubiquitin-activating enzyme, first conjugates ubiquitin's carboxyl terminus. This step is obligatory, and cell lines with temperature-sensitive ubiquitination have defects traced to mutations in E1 (Kulka, et al., 1988; Chowdary, et al., 1994). E1 transfers ubiquitin to an E2 which transfers it to an E3 which finally conjugates the ubiquitin to a target protein (sometimes, the E3 step is skipped). Most protein ubiquitination requires a single E1 protein, but E2 and E3 enzymes occur as families that regulate and confer specificity for ubiquitination. This arrangement explains why genetic defects in general ubiquitination only occur in E1 enzymes (Ciechanover and Schwartz, 1994; Hochstrasser, 1992).

Cells die without ubiquitination. The process has been implicated in a wide variety of cell functions reviewed elsewhere (Hochstrasser, 1996; Jentsch, 1992). Ubiquitination was originally discovered in the rapid degradation of short-lived and abnormal proteins by cytosolic proteases, and this role remains the best characterized (Hershko and Ciechanover, 1992). Heat stress causes enhanced degradation of long-lived proteins in E36 Chinese hamster lung cells. This heat-stress induced degradation occurs in lysosomes via autophagy and requires ubiquitin-activating enzyme E1 (Gropper et al., 1991; Handley-Gearhart et al., 1994). However, specific long-lived proteins that utilize this ubiquitin-mediated autophagic mechanism have not been identified.

Whether for short-lived or long-lived proteins, ubiquitin-mediated turnover involves attachment of multiple ubiquitins on a protein targeted for degradation (Hershko and Ciechanover, 1992). A ubiquitinated protein (a.k.a. ubiquitin conjugate) is

a substrate for further ubiquitination, and additional ubiquitins preferentially conjugate to already attached ubiquitin. A chain of ubiquitins is built on a protein to be targeted. The multiubiquitin chain then acts as a signal for degradation of the targeted protein. Each ubiquitin adds an additional 76 amino acids to the protein, and successive intermediates of multiubiquitination can be demonstrated as a ladder of bands on SDS-PAGE that contain both ubiquitin and the targeted protein (Chau et al., 1989; Hershko and Ciechanover, 1992). Multiubiquitination is a well-established signal for stress-induced degradation of short-lived proteins by a major protease complex in cytosol. Though total long-lived proteins undergo ubiquitin-mediated stress-induced degradation in lysosomes, a role for multiubiquitination has not been established for lysosomal degradation of any specific cytosolic protein.

Recently, work in the laboratory of William A. Dunn, Jr. demonstrated a connection between ubiquitin and the long-lived protein aldolase B. The evidence includes data presented in this dissertation, two meeting abstracts, and a manuscript which has been submitted (Lenk et al., Submitted 1998; Susan and Dunn, 1996; Susan et al., 1995). Together the data support that aldolase B is multiubiquitinated *in vivo* and suggest that ubiquitination is involved with stress-induced autophagic degradation of aldolase B in lysosomes.

S. E. Lenk and William A. Dunn, Jr. provided the first evidence that aldolase B has ubiquitinated forms (Figs. 1-3 and 1-4), including a major 68 kD form (Ub68) enriched in lysosomes during nutrient deprivation (Lenk et al., Submitted 1998; Susan et

Figure 1-3: Characterization of A Major Ubiquitin-Protein Conjugate Enriched in Autophagic Vacuoles. a) Rats were starved to induce autophagy and lysosomal uptake of ubiquitinated proteins. Subcellular fractions of liver were prepared, and equal protein from cytosolic (Cy), lysosome-enriched (Ly), and autophagic vacuole-enriched (AV) fractions were run on SDS-PAGE, western blotted, and labeled with antibody against a major ubiquitinated protein (anti-Ub68). Note major bands at 68 kD and cross-reactivity to a 40 kD protein in cytosol. The 40 kD protein was identified as aldolase B by peptide sequence analysis. b) Cytosol was circulated on an anti-Ub68 column, washed, eluted, and preparative SDS-PAGE performed. A gel strip was stained with Coomassie blue R-250 (CB), and the remaining gel was blotted and cut into strips individually stained with anti-Ub68 (Ub68) or anti-ubiquitin (Ub). Ub68 and aldolase are indicated at 68 kD and 40 kD, respectively. Arrowheads indicate positions of bands suggestive of intermediates of multiubiquitination of aldolase (from Lenk, et al., Submitted 1998).

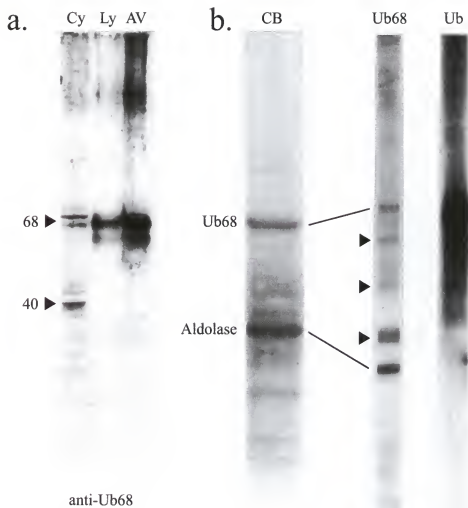
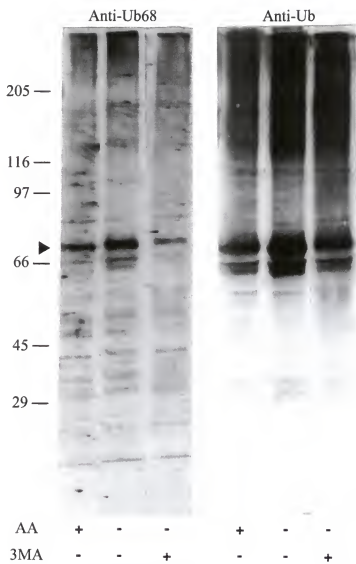


Figure 1-4: Amino Acid Starvation Increases Lysosomal Association of Putative Ubiquitinated Aldolase B via Autophagy. Fao rat hepatoma cells were incubated on media with or without amino acids (AA) and the autophagy inhibitor 3-methyladenine (3MA) as indicated. Sub-cellular fractions were collected, and equal protein of lysosome-enriched fractions were run on SDS-PAGE, western blotted, and stained with antibodies against Ub68 and ubiquitin (Ub). Positions of molecular weight standards and Ub68 (arrowhead) are indicated (from Lenk et al., Submitted 1998).



al., 1995). Aldolase A and B are long-lived proteins known to undergo degradation by autophagy (Henell et al., 1987; Kominami et al., 1983; Kuehl and Sumsion, 1970; Ueno and Kominami, 1991; Ueno et al., 1990). It has been determined that amino acid deprivation (starvation) rapidly enhances autophagy in cultured cells (Kopitz et al., 1990; Seglen and Gordon, 1982; Ueno et al., 1990). Since Ub68 increases in lysosomes under similar conditions (Fig. 1-4), the evidence suggested that ubiquitination might play a role in stress-induced autophagic degradation of aldolase B.

#### Hypothesis for Stress-Induced Degradation of Aldolase B

The field of protein degradation has made great progress in determining molecular mechanisms for the degradation of short-lived proteins via a cytosolic protease complex (the proteasome); however, long-lived proteins which are generally thought to be degraded in lysosomes have relatively poorly characterized degradative mechanisms. Cellular degradative mechanisms that respond to environmental changes facilitate experimental characterization. Starvation (amino acid and serum deprivation) and heat stress can induce regulated mechanisms for the degradation of long-lived proteins. Figure 1-1 presents two pathways proposed for the stress-induced delivery of cytosolic proteins to lysosomes for degradation: autophagy or receptor-mediated targeting (lower pathway).

In the simplified diagram of Figure 1-1, only topological changes in autophagy (upper pathway) are shown for a tetrameric cytosolic protein sequestered into an autophagic vacuole (steps 1 and 2) that fuses with lysosomes (step 3). Heat stress

induces autophagic degradation that requires ubiquitination, but specific proteins that are ubiquitinated during stress-induced autophagy have not been identified. Aldolase B is known to undergo autophagy, and a putative ubiquitinated form aldolase B associates with autophagic vacuoles and lysosomes during starvation. To establish a specific protein for ubiquitin-mediated autophagy, we examined aldolase B as a likely substrate for ubiquitin-mediated autophagy.

In Figure 1-1, receptor-mediated targeting to lysosomes is also drawn showing required components (lower pathway). Since established substrates for this pathway have conformations that would prevent receptor recognition, unknown factors (smallest circles) have been proposed to relax the structure of substrate proteins (first arrow) which probably includes disassembly of subunits from quaternary structures (rough-drawn oval with small circles attached). An exposed signal then mediates assembly of a complex on the lysosomal surface (second arrow), including the HSC73 chaperone (medium gray square), the substrate protein (extended coils), the lysosomal membrane protein LAMP2 (darkest rectangle), and possibly other factors (small circles). Transmembrane translocation (third arrow) also requires an intralysosomal HSP70 chaperone (dark gray square). Aldolase B has characteristics similar to known substrates for receptor-mediated targeting to lysosomes, but this mechanism was not examined for any aldolase. Evidence will be shown that ubiquitinated forms of aldolase B have a more denatured conformation. If aldolase B follows receptor-mediated degradation, then ubiquitin could represent the unknown factor needed to relax substrate structure.

The relationship between receptor-mediated targeting of cytosolic proteins to lysosomes and ubiquitin-mediated autophagic degradation had not been examined. Since aldolase B was a potential substrate for both pathways, I hypothesized that during stress, aldolase B requires both ubiquitination and a receptor-mediated targeting signal for enhanced degradation in lysosomes.

### General Strategy

I adopted the hypothesis that during stress, aldolase B requires both ubiquitination and a receptor-mediated targeting signal for enhanced degradation. The two requirements in this hypothesis were separately tested: ubiquitination and a receptor-mediated targeting signal. In this regard, there were two corresponding aims of this investigation: Aim #1, perturb ubiquitination and examine the effects on stress-induced delivery of aldolase B to lysosomes; Aim #2, mutate potential lysosomal targeting signals and examine effects on starvation-induced degradation of aldolase B.

My first aim was to determine whether stress-induced degradation of aldolase B requires ubiquitination. Antibodies were raised against aldolase B expressed in and isolated from *E. coli*. Since bacteria lack ubiquitin, the antibodies were produced against antigen that did not contain ubiquitin or ubiquitin-conjugated proteins. With the antibodies, the presence of aldolase B epitopes in a major 68 kD ubiquitinated protein (Figs. 1-3 and 1-4, Ub68) and other ubiquitin conjugates was confirmed in subcellular fractions from rat liver. Epitope-tagged aldolase B was expressed in E36 (parent) and ts20 (ubiquitination mutant) cells previously used to establish ubiquitin-dependency for

heat stress-induced autophagic degradation of long-lived proteins. By examining changes in the endogenous aldolase A and exogenous aldolase B associated with pelletable subcellular fractions, evidence was found that these aldolase isoforms require ubiquitination for autophagic degradation in lysosomes during heat stress. This supported my hypothesis that stress-induced degradation of aldolase B requires ubiquitination.

An attempt was made to use protein degradation measurements to confirm that heat stress-induced degradation of aldolase B requires ubiquitination. Degradation of aldolase B was found to utilize a temperature-dependent cytosolic proteolytic mechanism. The cytosolic proteolysis of aldolase B at heat stress temperatures was similar in magnitude to induced ubiquitin-mediated autophagic degradation. Since the cytosolic mechanism turned out to be ubiquitin-independent, degradation measurements could not confirm ubiquitin-mediated degradation of aldolase B via lysosomes. However, the results demonstrate that mechanisms for degradation of aldolase B include a novel cytosolic proteolysis.

My second aim was to test whether a receptor-mediated targeting signal was required for stress-induced degradation of aldolase B. A sequence motif has been defined for targeting cytosolic proteins to lysosomes for degradation during nutrient deprivation, and aldolase B contains three sequences that match the motif (Fig. 1-2). Depriving liver-derived cell lines of serum and amino acids causes starvation-induced degradation of long-lived proteins including aldolase B. Vectors were constructed expressing epitope-tagged aldolase B and used site-directed mutagenesis to disrupt the

putative targeting signals. Wildtype and mutant aldolase B proteins were expressed and assayed for starvation-induced degradation. Starvation causes enhanced autophagic degradation of aldolase B expressed in cultured hepatoma cells, and this enhanced degradation specifically required a targeting signal that includes glutamine residue #111. This supported my hypothesis that stress-induced degradation of aldolase B utilizes a receptor-mediated targeting signal.

## CHAPTER 2: MATERIALS AND METHODS

### Cell Lines and Culturing

#### General Maintenance

Except for temperature (see following subsections), all cell lines were maintained similarly using standard sterile cell culturing techniques. Except where indicated, all supplies were obtained from Fisher Scientific, Inc. The term “standard culture conditions” refers to maintenance in DMEM (Sigma #D-5648), 2.2 g/l NaHCO<sub>3</sub>, and 10% FBS (Biocell #6201-00) in a 5% CO<sub>2</sub> atmosphere, and the standard medium for stably transfected cells included 0.3 mg/ml active G418 (GIBCO BRL #11811-031). Cultures were fed every 3-4 days and passaged before complete confluency. For passages, cell sheets were rinsed with DPBS (Sigma #D-5652) followed by 1x trypsin-EDTA (Sigma #T4174) in DPBS for 4-8 minutes at room temperature or 37°C as needed. Passages to amplify and maintain cultures for experiments were split 1:10 to 1:50 (area:area), and very fast growing lines that tolerated thin splits were done down to 1:80. Since trypsin/EDTA diluted 1:10 or more with 10% FBS did not affect cell sheets during 30 min. at 37°C, some thin splits (at least 1:30 into medium with 10%FBS) were directly plated without pelleting to remove trypsin. For cultures using this short cut, attachment times, spreading times, growth rates, and experimental results were unaltered. Passages to replenish frozen stocks were always split heavily at 1:3 to 1:6 from freshly thawed stocks grown to near confluency. For new frozen stocks, cells

were suspended in medium supplemented with 10% DMSO (Sigma #D-2650), incubated 1-2 h at minus 20°C, then at minus 80°C overnight, and stored at minus 80°C for up to two months or transferred to liquid nitrogen for longer storage times.

#### Heat Stress-Inducible E36 Cells and Ubiquitination Mutant

Alan Schwartz kindly provided cell lines: E36 (parent), ts20 (mutant with temperature sensitive ubiquitin-activating enzyme E1), and ts20E1c2 (mutant rescued by wild-type human E1) Chinese hamster lung cell lines. These cells are well characterized for thermal control of ubiquitin-activating enzyme E1 activity and together have demonstrated that E1-mediated ubiquitination is required for heat stress-induced degradation of long-lived proteins ((Handley-Gearhart et al., 1994); (Handley-Gearhart et al., 1994); (Tausch et al., 1993); (Lenk et al., 1992); (Schwartz et al., 1992); (Gropper et al., 1991); (Kulka et al., 1988)).

#### Starvation-Inducible Cell Lines

William A. Dunn, Jr. provided Fao (rat hepatoma) and HuH7 (human hepatoma) cell lines. The Fao cell line originates from a rat hepatocellular carcinoma (Reuber, 1961), and this derivation is well documented (Deschatrette and Weiss, 1974). Fao cells retain a dozen liver-specific characteristics examined by Mary C. Weiss, including some endogenous expression of aldolase B (Deschatrette et al., 1979; Deschatrette and Weiss, 1974).

The HuH7 cell line was isolated from a well differentiated carcinoma of a Japanese man and shown to secrete 16 different plasma proteins associated with liver

function (Nakabayashi et al., 1982). Seven expected carbohydrate-metabolizing activities were present in HuH7 cells, but for two of these, liver-specific isoforms, pyruvate kinase L and a low- $K_m$  hexokinase, were not detected (Nakabayashi et al., 1982). BHK (baby hamster kidney), and NRK (normal rat kidney) cell lines were examined briefly during transient transfections.

### Plasmid Vector Construction and Mutagenesis

#### General Molecular Biological Methods

Basic methods were performed essentially as described in Current Protocols in Molecular Biology (Ausubel, et al., 1994). Except where noted, all supplies came from Fisher Scientific, Inc. Kits for DNA preparations were from Qiagen and Promega. Restriction digestions, ligations, other DNA modifications, and PCR utilized supplies from Promega and New England Biolabs, except as noted below.

#### PCR Primers and DNA Sequencing

At the University of Florida, the DNA Synthesis Core Laboratory provided all oligonucleotide primers that William A. Dunn, Jr. or Peter P. Susan designed for PCR. The University of Florida DNA Sequencing Core Laboratory sequenced parts of plasmid vectors that were altered, or we did DNA sequencing with a Sequenase™ kit (U.S. Biochemical Corporation).

#### Expression Vectors for Epitope-Tagged Aldolase B

Kiichi Ishikawa provided pRAB 1710 Amp<sup>+</sup> (Tsutsumi et al., 1984), a plasmid containing the cDNA of rat aldolase B (RAB) used as a PCR template in a reaction containing two primers (Fig. 2-1). PCR solutions were prepared according to

manufacturer's specifications (Promega) and then run through 40 cycles (each cycle: 1 min. at 94°C, 1 min. at 52°C, and 2 min. at 72°C), yielding DNA coding for rat aldolase B tagged with the 9E10 myc epitope at the carboxyl terminus (RABM). After restriction, the product was ligated into *Xho*I and *Xba*I sites of the vector pMAMneo-blue (CLONTECH), yielding pRABM which failed to express RABM. Using *Eco*RV and *Xba*I, I transferred the RABM code to pcDNA3 (Invitrogen), yielding pcDNA3RABM (Fig. 2-2). William A. Dunn, Jr. provided a pcDNA3-based vector, pHAHAB which expresses human aldolase B (Sakakibara et al., 1989) with an amino terminal 12CA5 HA epitope, HAHAB (Lenk et al., Submitted 1998).

- a. WID5, 5' primer: CTCCTTGGCTCGAGCTGTC

*Xho*I

*Xba*I

- b. WID9, 3' primer: TGCTCTAGACTActacaagtctcttcagaaataagctttgttcctcGTAGG-TGTAGGGGCTGTGA

Figure 2-1: Primers for PCR Amplification of Insert Containing cDNA for RABM Expression. a) 5' primer also called, WID5; b) 3' primer also called WID9. Orientations are relative to 5' to 3' convention. *Italics* indicate reverse complementary code for carboxyl terminal amino acids; lower case letters indicate reverse complementary sequence for myc (9E10) tag; single underline indicates reverse complementary sequence for a stop codon; boldface indicates *Xho*I and *Xba*I restriction sites; other bases are the same as vector sequences.

a. 5' DNA Sequence:

--TCTGCAGATATCAAGCTTATCGATACCGTCGACCCTCGAGCTGTCAATCA**TG**--  
EcoR V XhoI start  
methionine  
(aldolase B)

b. 3' DNA Sequence:

myc-epitope code  
 --ACCTACgaggaaacaaaagcttattttctgaagaagacttgTAGTCTAGAGGGCCC--  
 C-terminal stop *XbaI*  
 tyrosine codon  
 (aldolase B)

### C. Carboxyl terminal amino acids of RABM:

--TASYTYEEOKLISEEDL

**FIGURE 2-2: DNA Sequence of pcDNA3RABM.** a) 5' insertion site and b) 3' insertion site showing new sequence generated by vector construction. Boldface designates cDNA sequence of rat aldolase B (upper case) and human myc epitope 9E10 (lower case). Italics designate DNA sequence from the multicloning site of pMAMneo-blue™. Standard typeface designates DNA sequence in the multicloning site of pcDNA3™ (Invitrogen). Underline designates indicated restriction sites. Double underline designates indicated codons. Sequences not shown for rat aldolase B cDNA and pcDNA3™ vector are available in Tsutsumi, *et al.* (Tsutsumi et al., 1984) and from Invitrogen Technical Services, respectively. c) Amino acid sequence predicted for the carboxyl terminus of the RABM protein expressed from this vector (residues not shown would be identical to rat aldolase B), single letter amino acid code is underlined for residues added to create a 9E10 epitope.

### Site-Directed Mutagenesis of pcDNA3RABM

I used three different strategies for PCR mutagenesis: (1) overlap extension (Ho et al., 1989), (2) Quick-Change™ Site-Directed Mutagenesis Kit (Stratagene #200518), or (3) restriction-limited insertion as described below (Fig. 2-4; Table 2-1).

I used an overlap extension protocol adapted by Brian Cain from Ho and others (Ho et al., 1989). In brief, a mutagenic primer pair (see Fig. 2-3, positions 2 & 3, 4 & 5, or 6 & 7) was made complementary to each other and to base pairs on either side of a targeted change (non-complementary) in aldolase B coding sequence (Table 2-1). For example, Q58 mutagenesis started with pcDNA3RABM as template in two PCR reactions using primers at positions 1 & 2 (Fig. 2-3) to make product for the 5' end of an insert and at positions 3 & 8 (Fig. 2-3) to make product for the 3' end of an insert. PCR was done for 35 cycles (1 min. at 94°C, 1 min. at 55°C, and 2 min. at 72°C).

At one end of one product, sequence was derived from primer at position 2 and therefore was reverse complementary to one end of the other product derived from primer at position 3. These products were combined in Taq polymerase buffer (Promega), melted at 94°C, and cooled very slowly to allow promiscuous annealing and yielding a small amount of 3' end-to-end annealed single stranded DNA from 5' and 3' products for ends of a desired insert. Taq polymerase was added and the temperature raised to 72°C for run-off extension through ends complementary to positions 1 & 8 (Fig. 2-3). Primers for positions 1 & 8 were added and cycled through temperatures as done previously which produced a smear of products due to promiscuous annealing.

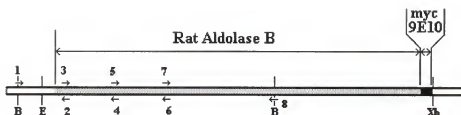


FIGURE 2-3: Positions of Primers for Site-Directed Mutagenesis. Map shows the RABM coding region of pcDNA3RABM; positions drawn to scale. Numbered arrows identify 5'→3' primer sequences at complementary sites. Shaded areas are cDNA sequences for the indicated polypeptides. Letters B, E, and Xb indicate restriction sites for enzymes *Bsa*I, *Eco*R V, and *Xba* I, respectively. E and Xb are sites of insertion into pcDNA3 (Invitrogen) multicloning site.

TABLE 2-1: Details of Primers for Site-Directed Mutagenesis

| Primer I.D. | Map Position* | Position (bp) From 1 <sup>st</sup> Base of Start Codon | Target Code Change         | Primer Sequence, 5' to 3' (sequence non-complementary to pcDNA3RABM is underlined) |
|-------------|---------------|--------------------------------------------------------|----------------------------|------------------------------------------------------------------------------------|
| WID17       | 1             | -117 to -91                                            | + <i>Sma</i> I             | CTCACTATAGGGAGACCC <u>GGG</u> CTTGGT                                               |
| WID19       | 2             | +25 to +45                                             | Q12(T/N)                   | CTCCTTCTTA(T/G)TCTCTGAGGT                                                          |
| WID18       | 3             | +25 to +45                                             | Q12(T/N)                   | ACCTCAGAGA(A/C)TAAGAAGGAG                                                          |
| WID21       | 4             | +163 to +181                                           | Q58(T/N)                   | CTCGGA(T/G)TCCTTCGGTT                                                              |
| WID20       | 5             | +163 to +181                                           | Q58(T/N)                   | AACCGAAGGA(A/C)TTCCGAG                                                             |
| WID44       | 4-non***      | +165 to +195                                           | + <i>Sca</i> I<br>Q58N     | <u>AGAGAAGT</u> ACTAAAGAGGAGCTCTCGG<br>AAATTCCTTCGG                                |
| WID43       | 5-non***      | +196 to +218                                           | + <i>Pml</i> II            | <u>CGAGACAC</u> GTGGACAATTCTATC                                                    |
| WID23       | 6             | +322 to +339                                           | Q111(T/N)                  | ACCTCCA(G/T)TGTCAGCTT                                                              |
| WID22       | 7             | +322 to +339                                           | Q111(T/N)                  | AAGCTGGACA(A/C)TGAGGT                                                              |
| WID36       | 6             | +313 to +340                                           | Q111T<br>+ <i>Hind</i> III | CACCTCCTGTGTCAAGCTTGATGCCCAC                                                       |
| WID35       | 7             | +313 to +340                                           | + <i>Hind</i> III<br>Q111T | GTGGGCATCAAGCTTGACA <u>C</u> AGGAGGTG                                              |
| WID24       | 8             | +617 to +636                                           | none                       | AGCAGCCAAGACCTTCTCAG                                                               |

\*Fig. 2-3; \*\*amino acid (single letter code) change by residue # (start M = 1); \*\*\*primer non-complementary to paired primer with position shifted for restriction-limited insertion (Fig. 2-4).

Though extraneous products were common, the most abundant product was the desired mutated DNA fragment representing full length mutated insert. This insert containing altered DNA code was *BsaI* digested, gel purified, and ligated into the corresponding site of fresh pcDNA3RABM from which the wild type fragment was removed.

Normally, restriction sites for primer positions 1 & 8 would be designed for two unique-site enzymes producing different overhangs. However, *BsaI* cuts outside its recognition sequence producing randomly unique overhangs that abrogate a need for separate enzymes. *BsaI* cuts a third site near the ampicillin-resistance gene of pcDNA3RABM, producing two fragments of vector besides the insert fragment. Overhangs for all three sites were randomly different allowing three-fragment ligation with proper orientations.

The Quick-Change™ Site-Directed Mutagenesis Kit (Stratagene) was also used and found to be more rapid. The manufacturer's protocols were followed, and primers designed for primer extension and amplification at Q111 (WID23 and WID22 at positions 6 & 7) did not work with the Quick-Change™ kit. However, longer primers (WID36 and WID35) were successful. The Quick-Change protocol involves *in vitro* synthesis of the entire vector (6.5 kb), possibly introducing errors anywhere in mutated pcDNARABM. To reduce sequencing, the *BsaI* fragment containing new mutations was cassetted into fresh vectors. All altered regions of vectors were sequenced at least twice to confirm changes in amino acid coding were specific for targeted residues.

For Q58N mutation, a restriction-limited insertion was designed(Fig.2-4). This method uses a mutagenic primer (WID44) to span the Q58 codon and code for unique

blunt-end restriction site, *ScaI* (restriction-limited). Another primer (WID43) was designed with another unique blunt site, *PmlI*, such that *ScaI* to *PmlI* blunt ends ligated to make proper aldolase B code. Steps were followed as indicated in Figure 2-4 to produce an expression vector for the mutated RABM, pcDNA3RABMQ58N.

### Expressing Epitope-tagged Aldolase B in Cell Lines

#### Permanent Lines Expressing RABM

E36 and ts20 cells were transiently transfected with pcDNA3RABM, using Lipofectin (GIBCO BRL) or DOTAP (Boehringer) by the manufacturers' protocol. When transfected cultures approached confluency labeled cells occurred in groups of 2 to 8 presumably due to cell division. Transfected cultures were trypsinized at confluency and diluted >1:15 by area into G418 Medium. Fresh G418 Medium was provided every 1-2 days as needed to remove cell debris and maintain strong selection. By 2-3 weeks post plating, colonies of resistant cells were isolated, passaged, and screened for RABM expression (immunofluorescence microscopy and western blotting with anti-9E10 monoclonal antibody as described below).

Different lines permanently expressed RABM at varied levels (~10 fold range on western blots). Using experimental protocols described below, no effect was seen with doubling time, degradation of RABM, degradation of total protein, or viability. Of eight positive clones that continue to express after culture amplification, seven (4 from E36 and 3 from ts20) maintained stable relative levels of RABM for twenty additional continuous doublings or longer (by immunofluorescence and on western blots, data not

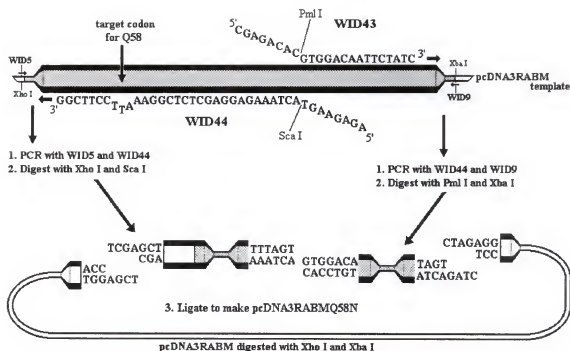


Figure 2-4: Restriction-Limited Insertion for Constructing pcDNA3RABMQ58N.

Above, primers (small arrows and sequences for WID43 and WID44) are shown relative to pcDNA3RABM template (bar). Sequences juxtaposed to template indicate complementary regions. Relative positions of Q58 codon and restriction sites are indicated. Below, DNA pieces for a three-fragment ligation. See text, Fig. 2-3, and Table 2-1.

shown). To facilitate experimental quantification, the highest RABM-expressing E36 and ts20 cell lines fully designated E36RABM14.1 and ts20RABM10.2 or abbreviated throughout this dissertation as E36AB and ts20AB, respectively, were used. In control experiments, variation in the level of RABM expression did not affect results (data not shown).

Similar procedures were repeated with Fao rat hepatoma cells transfected, except another plasmid pHAHAB also was used to express human aldolase B tagged with the 12CA5 HA epitope at its amino terminus (HAHAB). Screening for HAHAB expression was done with monoclonal antibody against 12CA5. When G418-resistant clones were isolated at very most 20% of cells in a given clone had visible expression that was mostly dim with a few bright cells, and this fraction was rapidly lost with culture splitting for amplification. Subcloning and screening of a few hundred colonies produced one truly stable line expressing HAHAB at levels comparable to ts20AB expression of RABM. This line was designated FaoAB. FaoAB cells split at low density (1:20 or less) grew much slower than parent Fao cells. In passages using about 1:10 or 1:15 splits, growth rates were similar to parent Fao cells. An attempt to isolate HuH7 cell lines expressing RABM was made, but failed to produce any clones having permanent expression.

#### Transient Transfection System

Fao, HuH7, NRK, and BHK (hepatic and renal cell lines derived from tissues that express aldolase B) were transfected for transient RABM expression with a series of lipids according to manufacturer's standard protocols (Invitrogen and Boehringer-

Mannheim). Transient transfection gave very broad cell-to-cell variation in expression by 9E10 immunofluorescence, but ratios of bright to dim cells were relatively reproducible between transfections. Transfection efficiency was defined as fraction of labeled cells.

#### Immunofluorescence

Cells were grown on glass coverslips to desired confluency, rinsed briefly with PBS and fixed with 4% paraformaldehyde in PBS for 20-30 minutes. Fixed cells were washed three times for 10 minutes in 50 mM ammonium chloride/ 0.1% Tx-100/PBS. Coverslips were placed on drops containing antibody diluted 1:100 in 5%NGS/ 0.1% Tx-100/PBS for 1-2 hours at room temperature. Coverslips were washed four times for 5 minutes in 0.1% Tx-100/PBS. Coverslips were placed on drops containing an appropriate secondary antibody (rhodamine or fluorescein conjugated) diluted 1:100 in 5%NGS/ 0.1% Tx-100/PBS for 1 hour at room temperature. Coverslips were washed six times for 5 minutes in 0.1% Tx-100/PBS, then mounted on Fluoromount G (GIBCO BRL).

#### Antibodies

##### Preparation of Ubiquitin-Free Aldolase B Antigen

Different *E. coli* strains were transformed with pXPB, a plasmid vector kindly provided by Dean R. Tolan for bacterial expression of enzymatically active human aldolase B (Beernink and Tolan, 1992). The aldolase B expressed in *E. coli* retains all the enzymatic properties of the protein isolated from human liver (Sakakibara et al.,

1989). Increased expression of a 40 kD Coomassie signal in SDS-PAGE of whole cell preparations was apparent in transformed cells.

*E. coli* JM83 cells were transformed with pXPB, which produced much more 40 kD protein than untransformed cells, about 0.3 mg per ml of 1.9 OD<sub>600 nm</sub> culture. A 250 ml culture of LB broth + ampicillin (100 µg/ml) was grown to 1.9 OD<sub>600 nm</sub> culture and pelleted in a Beckman GSA rotor at 3500 rpm for 10 min. Samples were maintained at 0-4°C for the rest of the procedure. The cell pellet was resuspended in 20 ml 15% sucrose/50 mM EDTA/50 mM Tris-HCl pH 8.5. To this, 5 ml 5 mg/ml lysozyme was added, gently mixed by inverting, and incubated 15 min. Then 15 ml 0.1% Triton X-100/50 mM Tris-HCl pH 8.5 was added, gently mixed, and incubated with periodic inverting for 20 min. After centrifugation at 9000 rpm for 30 min. (GSA rotor), the supernatant was decanted into Polyclear tubes and ultracentrifuged in aSW27 rotor at 23,000 rpm for 60 min. The resulting supernatant was the crude extract which was further processed as previously described for isolation of aldolase B from liver extracts (Penhoet and Rutter, 1975). Saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was slowly added (0.5 ml/min.) to 45% final concentration with constant stirring. After centrifugation at 9000 rpm for 60 min., the supernatant was collected and the pellet discarded. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was slowly added to 60% concentration, and 6 N NH<sub>4</sub>OH added to pH 7.5. The mixture was immediately transferred to centrifuge bottles and let stand for >2 hours. After centrifugation at 9000 rpm for 60 min., the 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> pellet was dissolved in 1 mM EDTA/10 mM Tris-HCl pH 7.5 and dialyzed against the same buffer. The sample

was loaded onto a 25 ml phosphocellulose (fine mesh, 1.26 meq/g) column prepared exactly according to Penhoet and Rutter. The column was washed with 5 mM EDTA/50 mM Tris-HCl pH 7.5 (50-60 ml) until  $OD_{280\text{ nm}}$  approached zero. Then 2.5 mM fructose 1,6- diphosphate in 1 mM EDTA/10 mM Tris-HCl pH 7.5 was used to specifically eluted a sharp aldolase peak. Peak fractions with specific activities of 0.83 to 0.98 aldolase U/mg (1 U/mg expected for aldolase B) were pooled, precipitated by 55%  $(\text{NH}_4)_2\text{SO}_4$ , and stored as a suspension at 4°C. Before immunization, the suspension was dialyzed into 10 mM Tris-HCl pH 7.5. According to Coomassie labeled SDS-PAGE and enzymatic properties (Rutter et al., 1966), the recombinant human aldolase B constituted at least 95% of the final protein and was more than 99.99% pure of bacterial aldolase activities, containing  $\leq 0.005\%$  bacterial isoform (EDTA-sensitive) activity.

#### Production of Antibodies Against Aldolase B

Rabbits were fed and housed by University of Florida Laboratory Animal Services. Antibodies to native and denatured aldolase B were raised as previously described (Reznick et al., 1985) except ubiquitin-free human aldolase B antigen was used (as prepared above). For making antibody to native aldolase B, 50  $\mu\text{g}$  antigen in 0.5 ml 10 mM Tris-HCl pH 7.5 was emulsified 1:2 with complete and incomplete Freund's adjuvants for immunizations and boosts, respectively. For antibody to denature antigen, the antigen solution was supplemented with 2%SDS/2% $\beta$ ME and boiled 10 min. prior to mixing with adjuvant and immediately before administration to animals. Intradermal

injections were in the thoracic region on the backs of specific pathogen-free New Zealand White rabbits. The first boost was two weeks after initial immunization.

Preparative Western blots of rat liver cytosol were routinely used to follow specific immunoreactivities. One week after the first boost, the rabbit receiving native antigen produced a highly reactive serum specific for 40 kD aldolase B which was maintained without further boosts. Unless otherwise specified, these antibodies were used to detect native aldolase B-specific polyclonal epitopes throughout this study.

One week after the first boost, the rabbit receiving denatured antigen produced antibody that specifically labeled a 68 kD protein moderately, 60 kD and 78 kD proteins lightly, and a high molecular weight smear. This pattern was remarkably similar to that for anti-Ub68. Furthermore, relative recognition of isoforms A and B were comparable to that for Ub68. Thus, denaturation of aldolase B disrupted isoform-specific epitopes and produced anti-denatured aldolase B that similarly recognized epitopes in both aldolases A and B. Interestingly, the early bleeds had little or no reactivity to 40 kD aldolase.

Every 4-7 weeks (after injection sites completely healed), the rabbit immunized with denatured aldolase B was boosted. After the second boost, antibody immunoreactivities were greatly increased. Though these sera recognized some non-protein epitopes, they retained specificity for proteins labeled with sera from earlier bleeds. After the third boost, sera recognized 40 kD aldolase B but never as well as antibody to native antigen.

### Other Antibodies

Anti-Ub68 was provided by William A. Dunn, Jr. and is a polyclonal rabbit antibody raised against a major ubiquitin-protein conjugate purified from lysosomes (Lenk et al., Submitted 1998). Monoclonal mouse antibodies against the12CA5 HA and 9E10 myc epitopes were obtained from the University of Florida Hybridoma Core Laboratory. Alternatively, hybridoma cells expressing anti-9E10 (provided by the Hybridoma Core) were injected into the peritoneum of BALB/c mice (provided by the University of Florida Laboratory Animal Services Division), and periodically, ascites was harvested until mice showed signs of discomfort or disease at which time they were euthanized.

### Viability Assays

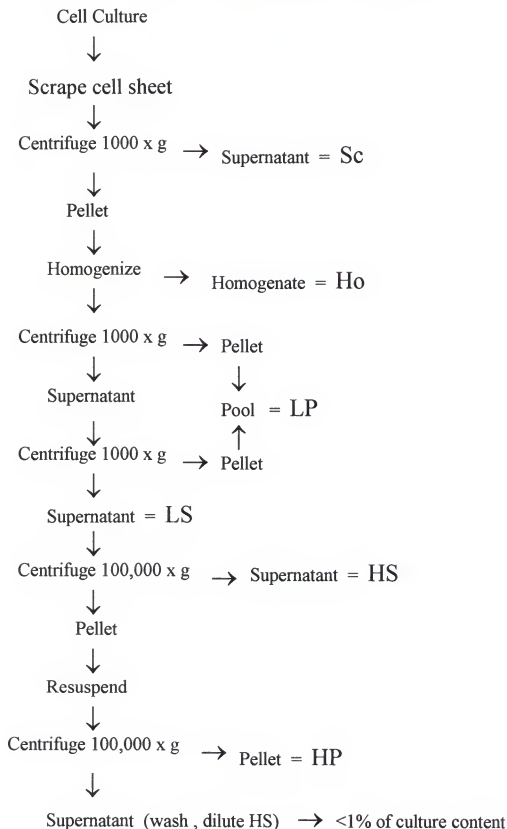
All treatments were measured for viability except for incubations in HBSS (Hank's Balanced Salts Solution) which were used to induce cell death. Heat stressed cultures using HBSS had many cells rounding and sloughing off culture surfaces in 4 to 6 hours of treatment, and were only used in experiments to determine the effect of dying cells on protein degradation measurements. When HBSS was replaced with MEM (Sigma #M-0268 + 2.2 g/l sodium bicarbonate), such rounding and sloughing was delayed beyond 25 hours of treatment. To measure metabolic viability, cultures exposed to each experimental treatment were recovered under normal maintenance conditions for 12 hours followed by addition of the same labeling medium (0.1 mCi  $^{35}\text{S}$ -methionine/ml) used for protein degradation assays. Then protein synthesis was measured as TCA

precipitable counts incorporated in 20 min. Metabolic viability was defined as the % protein synthesis relative to duplicate cultures treated with fresh medium under normal maintenance conditions. Since experimental treatments (see Experimental Conditions) lack serum and nutrients (MEM instead of DMEM) relative to maintenance conditions, our assay probably underestimates metabolic viability. In MEM, more than 80% of metabolic viability was retained for 28 hours and 15 hours in heat stressed E36 and ts20 cells, respectively. Unless otherwise stated, all data are reported for incubations and drug treatments that retained 90% or greater metabolic viability. For low density cultures (<10% confluent), the Cell Titer 96 AQ System® was used according to manufacturer's protocols (Promega) which indirectly measures electron transport pathway activity.

#### Subcellular Fractionation

Subcellular fractionation of E36 and ts20 cells is summarized in Figure 2-6. To produce a homogenate containing intact organelles, cells were grown to recent confluency in 100 mm dishes, using 10 ml DMEM (Sigma #D-5648) + 10% FBS medium. Culture rinsed with 10 ml DPBS (Sigma D-5652) was treated with 1.0 ml 1 X Trypsin-EDTA (Sigma #T-9395 diluted in DPBS), and incubated at room temperature until cells easily and completely knocked loose from the plate (10 minute maximum). After adding, 5 to 10 ml medium containing 10% FBS, cells were transferred to 15 ml conical centrifuge tubes (polypropylene), centrifuged at 1,500 X g for 5 minutes, and supernatant discarded. Pellet was suspended in 1.0 ml ice-cold cavitation buffer (SHE):

Figure 2-6: Subcellular Fractionation Scheme for E36 and ts20 Cells. 50



250 mM sucrose, 10 mM HEPES (Research Organics #6003H-3), 1 mM EDTA, pH 7.4, loaded into a N<sub>2</sub>-cavitation bomb chamber using 2-3 ml total SHE volume, pressurized to 65 psi for 10 minutes, and collected sample from bomb directly into a pre-chilled Dounce homogenizer. Procedural details were as described by the cavitation bomb manufacturer's specifications (Kontes). The sample was homogenized with 5 strokes of a pestle, minimizing froth by limiting passage of bubbles to the sample side of pestle. This was saved as the homogenate (Ho).

Alternatively, the trypsinization step was replaced by scraping the cell sheet directly into SHE which resulted in a large fraction of cytosol but not organelles to leak out. This facilitated the separation of aldolase associated with organelles from soluble aldolase in the cytosol. Scraped cells were pelleted as done above to remove trypsin solution, but in this case the supernatant was saved (to assess cytosolic leakage) as the scrape fraction (Sc). The rest of cavitation and homogenization was performed as above.

Homogenate (Ho) was fractionated essentially as described previously (Rickwood, 1992; Coligan et al., 1995), using Sigma reagents for assays and Sorvall or Beckmann centrifuges and accessories. Homogenate was centrifuged at 1,500 X g for 20 minutes yielding a low speed pellet (LP) and supernatant. The centrifugation was repeated with the supernatant to make sure nuclei, large debris, and unbroken cells were efficiently removed, the resulting pellet was pooled in LP, and the resulting low speed supernatant (LS) was centrifuged at 100,000 X g for 90 minutes, yielding a high speed

supernatant (HS) and pellet. The pellet was resuspended in well over 400 volumes of fresh SHE and centrifuged at 100,000 X g for 60 minutes. The supernatant had a content similar to HS but was much more dilute (data not shown), so it was not pooled with HS. The pellet was saved as the high speed pellet (HP).

Fractionation conditions were developed to separate abundant cytosolic aldolase from that associated with organelles. An initial scrape into fractionation buffer caused a three fold greater leakage of aldolase than acid phosphatase, so this step was retained in the procedure. Conditions were chosen to maximize recovery of lysosomal organelles (acid phosphatase and  $\beta$ -hexosaminidase) from LP to HP and minimize release of lysosomal markers into Sc and HS. Recovery was reasonable (85-99% accounted).

### Enzyme Assays

#### Aldolase

Aldolase assays were performed as previously described (Penhoet and Rutter, 1975). "Aldolase reaction mix" includes 50  $\mu$ l 6.3 mg/ml  $\alpha$ -glycerophosphate dehydrogenase-triose phosphate isomerase mixture + 4 mg NADH (99% pure, Sigma) + 20 ml 0.1 M glycylglycine pH 7.5. Add 5-50  $\mu$ l of sample to 1 ml of aldolase reaction mix, and measure background  $\Delta$ OD (340 nm)/min. (BG); add 50  $\mu$ l 50 mM fructose-1,6-diphosphate (FDP) or 100 $\mu$ l 100 mM fructose-1-phosphate (F-1-P), and measure assay  $\Delta$ OD (340 nm)/min. (ASSAY). Aldolase activity in units,  $U = (ASSAY - BG) / 12.44$  for FDP or  $= (ASSAY - BG) / 6.22$  for F-1-P.

### Acid Phosphatase

Acid Phosphatase activity was the OD (405 nm) in 1000x g supernatant after 60 minute incubation at 37°C for 50 µl sample in 200 µl 8 mM p-nitrophenolphosphate/ 2mM MgCl<sub>2</sub>/ 90 mM Na-acetate pH 5.0 stopped by 600 µl 0.25 M NaOH (Rickwood, 1992).

### Protein Analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), gel staining and drying, western blotting, and autoradiography followed standard protocols similar to those described and referenced elsewhere (Ausubel et al., 1994; Coligan et al., 1995). Gels were made with 10% polyacrylamide (1:35 bis-acrylamide). Immunochemiluminescent detection was done with Amersham ECL Reagents and protocols, except blots were rinsed in Kodak 1XCDs buffer just before exposure to ECL chemicals. Protein concentrations were determined with Bio-Rad protein assay (IgG standard) reagent or spectrophotometric absorbance (205 nm or 280 nm).

For direct immunoprecipitations, monoclonal antibody was purified and conjugated to sepharose 4b by a CNBr protocol similar to that described in Coligan et al., 1995. Cells were incubated on a shaker at 0-4°C with ice cold minimum lysis buffer (MLB: 1%NP40/ 1 mM EDTA/50mM Tris-HCl pH 7.4), standard lysis buffer (SLB: 150 mM NaCl/ MLB), or modified radioimmunoprecipitation assay buffer (mRIPA: 0.1% SDS/ 0.25% desoxycholate/ SLB) supplemented with a cocktail of protease inhibitors (leupeptin, TLCK, pepstatin, aprotinin, and PMSF obtained from Sigma or

Boehringer/Mannheim) at concentrations according to Harlowe. The mRIPA gave the best results with mAb's conjugated to sepharose, and SLB gave the best results with polyclonal sera precipitated with commercial protein A-agarose (Boehringer-Mannheim). Lysates were precleared for 15-20 minutes on maximum in a microfuge then transferred to tubes containing 4-10  $\mu$ l packed bed volume of mAb-sepharose or 1-5  $\mu$ l antiserum then rotated in the cold. After one hour, tubes containing antiserum only received 10  $\mu$ l packed bed of protein A-agarose. Rotating incubation was continued for varying times, usually overnight, and such times were held constant within a given experiment. Rapid washes by quick microcentrifugation and aspiration of supernatants were done three times with the same lysis buffer and one time with TBS, then immediately processed for other procedures. For SDS-PAGE, a 2-fold concentration of sample buffer was directly applied to the pellet and heated at 95-100°C for 5-10 minutes. For precipitable aldolase activity, pellets were further washed with TE pH 7.5 then resuspended in aldolase reaction buffer.

Densitometry (autoradiographs, chemiluminographs, & Coomassie gels) were quantified using a desktop scanner and Sigmagel software. Automatic brightness and contrast settings determined initial settings that were then kept constant. Spot settings were chosen to encompass regions of interest, reduce background, and optimize signal. Standard curves were routinely performed to characterize linear response ranges for relative protein levels which also defined backgrounds and allowed quantification of relative signals. Where protein bands were specific for transfected cell lines,

untransfected cells were run in parallel through procedures and quantified to establish backgrounds.

### Stress-Induction of Protein Degradation

#### Culture Preparation

Cells were plated, grown, and maintained at confluency for 2-3 days. About 30-40 hours before an experiment, cultures were fed with standard maintenance medium or for protein degradation assays,  $^{14}\text{C}$ -Valine or  $^{35}\text{S}$ -Methionine was made up in comparable medium with the corresponding unlabeled amino acid omitted. At the beginning of an experiment, cells were switched to the media and temperatures indicated in Table 2-2.

| <b>Table 2-2: Comparison of Systems for Stress-Induced Degradation of Proteins</b> |                              |               |                             |               |
|------------------------------------------------------------------------------------|------------------------------|---------------|-----------------------------|---------------|
|                                                                                    | <b>Heat Stress Induction</b> |               | <b>Starvation Induction</b> |               |
|                                                                                    | <b>Control</b>               | <b>Stress</b> | <b>Control</b>              | <b>Stress</b> |
| <b>Medium</b>                                                                      | MEM                          | MEM           | DMEM+FBS                    | KH*           |
| <b>Temperature</b>                                                                 | 30.5°C                       | 39.5°C        | 37°C                        | 37°C          |

\*KH, Krebs-Heinseleit medium (Lefer et al., 1982)

#### Heat Stress

Cultures were prepared as described above and replicates were incubated under control temperature (CT) and heat stressed (HS) conditions (Table 2-3) which are permissive and non-permissive, respectively, for the ubiquitin-activating enzyme E1 of ts20 cells (Handley-Gearhart et al., 1994; Kulka et al., 1988; Lenk et al., 1992). Accordingly, CT conditions included 4 mM bicarbonate-buffered MEM under 5%  $\text{CO}_2$  at 30.5°C, and HS conditions included 20 mM HEPES-buffered MEM under air at 41.5°C for 1 hour followed by 39.5°C. These incubations followed established protocols for the

E36/ts20 cell system, except MEM replaced HBSS to improve viability. In some experiments, media were supplemented with 5-10 mM 3-methyladenine (3MA) or 40-160  $\mu$ M chloroquine (CHQ) as indicated in Results. Logistics and consistency with published protocols required differences in buffering and atmosphere between CT and HS conditions. As a control for such

| <b>Table 2-3: Heat Stress (HS) and Control Temperature (CT) Treatments to Determine Ubiquitin-Activating E1 Mediated Processes</b> |                                       |                                |                                |
|------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|--------------------------------|--------------------------------|
| <b>Experimental Condition</b>                                                                                                      | <b>MEM buffer*</b>                    | <b>Temperature</b>             | <b>Incubator</b>               |
| <b>CT (Permissive)</b>                                                                                                             | 2.2 g /l NaHCO <sub>3</sub><br>pH 7.4 | 30.5°C                         | standard<br>5% CO <sub>2</sub> |
| <b>HS (Non-permissive)</b>                                                                                                         | 20 mM HEPES<br>pH 7.4                 | 1 hour @ 41.5°C<br>then 39.5°C | submerged<br>in water bath     |
| <b>Repeat treatments with two cell lines: E36 (parent) and ts20 (mutant)</b>                                                       |                                       |                                |                                |

\*For protein degradation experiments, medium was supplemented with unlabeled amino acid corresponding to that used for labeling (5 mM methionine or 10 mM valine). differences, CT as summarized in Table 2-3 was compared with CT in HEPES-buffered

MEM under air, yielding no differences in cell morphology, viability, or protein degradation measurements (data not shown). The other control (*i.e.* comparing HS in conditions above with HS in bicarbonate-buffered MEM under 5% CO<sub>2</sub>) was not tested, because bicarbonate buffering varies with temperature.

#### Starvation (Nutrient Stress)

Cultures were prepared as described above and replicates were refed with fresh standard maintenance medium (DMEM + 10 % FBS) or Krebs-Heinseleit (KH) medium

and referred to as “Fed” or “Starved,” respectively. KH components are given in Lefer, et al., 1982. For protein degradation experiments, media were supplemented with unlabeled amino acid corresponding to that used for labeling (5 mM methionine or 10 mM valine). Inhibitor treatments utilized the same levels as for heat stress above.

### Protein Degradation

Permanent cell lines or transiently transfected cultures were treated according to instructions in the section Stress-Induction of Protein Degradation to produce cells with metabolically labeled proteins containing  $^{35}\text{S}$ -methionine or  $^{14}\text{C}$ -valine. Cell sheets were routinely rinsed with DPBS (Sigma) immediately followed by application of media containing unlabeled excess amino acid (5 mM methionine or 10 mM valine) to cultures described above. This initiated chase of radiolabel incorporated into proteins.

At various times, aliquots of media were collected and TCA precipitated to measure release of soluble counts measured with a scintillation counter. At the end of the chase, whole cultures were TCA precipitated to determine total counts. Fraction of TCA soluble counts released at various times were subtracted from 1 to calculate TCA precipitable counts, representing the remaining total radiolabeled protein at those times.

Alternatively, cultures were harvested at each time point, processed for immunoprecipitation, SDS-PAGE, and autoradiography, and the radioactive signals in specific protein bands (absent in untransfected cells) were quantified by densitometry, representing the remaining radiolabeled protein (aldolase) at those times.

To compare degradative rates for proteins expressed at different concentrations, relative rates are normalized to the initial amount of the protein, giving a fractional change per time with units %/hour. This fractional rate is constant for unchanging degradative mechanisms regardless of a substrate protein's concentration. For a given protein, this fractional rate defines the degradative rate constant,  $k_d$ . For total proteins, the fractional rate represents weighted average  $k_d$  contributed by the varied amounts of different proteins. Throughout this dissertation, all degradative rates and other rates of protein decrease are estimated with the following calculation. Fractions of radiolabeled protein remaining were transformed by the natural log (ln) and regression analysis performed using the following function:

$$\ln(100 \cdot S_t / S_0) = -k_d \cdot t,$$

where  $S_t$  = signal at time  $t$ ,  $S_0$  = initial signal,  $k_d$  = first order degradative rate constant, and  $t$  = time. Degradation rates were taken as the negative slope of the regression ( $k_d$ ) and the standard error of the slope was calculated as the standard error of  $y$  at  $x$  divided by the square root of the deviations squared of  $x$ . Degradative turnover is also described by half-life,  $t_{1/2}$ , the time needed to replace 50% of existing molecules with new ones. In general, the degradative rate constant and half-life are simply converted by  $k_d = \ln(2) / t_{1/2} = 0.693 / t_{1/2}$ . Note that some investigators do not follow the empirically confirmed first order relationship of  $k_d$  and  $t_{1/2}$ . This results in  $k_d$  and  $t_{1/2}$  reported in the literature that can vary by as much as three fold from similar data presented using conventional calculations.

## CHAPTER 3: UBIQUITINATION MEDIATES LYSOSOMAL PROTEOLYSIS OF ALDOLASE B

### Introduction

Aldolase B undergoes degradation during stress via autophagy. In Chapter 1, evidence was described for ubiquitin protein conjugates which underwent starvation-induced enrichment in autophagic vacuoles and lysosomes, and preliminary work suggested that putative ubiquitinated aldolase B was amongst these conjugates (Figs. 1-3 and 1-4). This resulted in the hypothesis that stress-induced autophagic degradation of aldolase B requires ubiquitination. In the first part of this chapter, the presence of aldolase B in ubiquitin conjugates is confirmed, and a role for ubiquitination in the heat stress-induced autophagic degradation of aldolase B is demonstrated.

Two mechanisms were found to simultaneously mediate enhanced degradation of aldolase B during heat stress: autophagy and cytosolic proteolysis. To separately examine effects on autophagy, heat stress-induced changes in endogenous aldolase A and exogenous epitope-tagged aldolase B associated with pelletable organelles were assayed. In addition, autophagic degradation of long-lived proteins was demonstrated to require ubiquitination. The results described below support a role for ubiquitination in the function of a subset of lysosomal proteases.

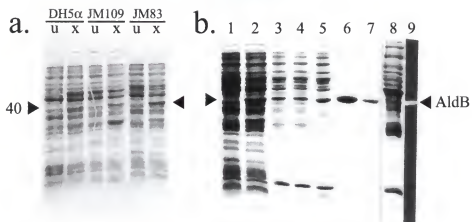
### *In Vivo* Multiubiquitination of Aldolase B

Previously, antibodies were raised to a major ubiquitin-conjugated protein, Ub68, that associated with autophagic vacuoles and lysosomes during stress-induced autophagy. On western blots of subcellular fractions isolated from rat liver, anti-Ub68 produced a pattern suggestive of a 40 kD protein successively conjugated with serially increasing numbers of ubiquitin. Such a pattern is referred to as a ubiquitin ladder for the modified core protein. Peptide sequence analysis identified the 40 kD protein as aldolase B, suggesting that anti-Ub68 reactive proteins might represent a ubiquitin ladder for aldolase B.

To confirm the existence of ubiquitinated forms of aldolase B, antibodies (Fig. 3-2) were raised against aldolase B (Fig. 3-1) and used to assay western blots of rat liver fractions from the previous studies (Fig. 3-3). In short, anti-aldolase B recognized the same proteins as anti-Ub68, confirming a ubiquitin ladder for aldolase B and demonstrating that aldolase B is multiubiquitinated *in vivo*. The data are consistent with ubiquitin-mediated autophagic degradation of aldolase B.

First, antibodies against aldolase B had to be produced. However, aldolase B isolated from animal cells would need to be purified away from contaminating ubiquitin and ubiquitin conjugates. Since bacteria lack ubiquitin, aldolase B was expressed in *E. coli* and purified to produce ubiquitin-free antigen for immunization (Fig. 3-1). *E. coli* strains transformed with an expression vector for human aldolase B (Fig. 3-1a, lanes x) produced more 40 kD protein (expected size of aldolase B) relative to untransformed cells (Fig. 3-1a,

Figure 3-1: Isolation of Ubiquitin-Free Aldolase B Expressed in *E. coli*. a) Three *E. coli* strains DH5 $\alpha$ , JM109, and JM83 were transformed for aldolase B expression as described in the text, pelleted, suspended in sample buffer, boiled, and run on SDS-PAGE with 60  $\mu$ l culture ( $OD_{600\text{ nm}} = 1.5$ ) equivalents per lane, u and x designate untransformed and transformed cells, respectively. The 40 kD band specific for transformed cells is indicated; b) *E. coli* JM83 cells expressing aldolase B were fractionated as described in the text: 1 and 2, whole cell preparations (as in part a) untransfected and transfected, respectively; 3, crude extract; 4, 45%  $(\text{NH}_4)_2\text{SO}_4$  supernatant; 5, 45-60%  $(\text{NH}_4)_2\text{SO}_4$  cut; 6, aldolase activity peak from phosphocellulose column; 7, dialyzed antigen ready for immunization; 8, rat liver cytosol used to screen antibodies; 9, detection of lane 8 with antibody raised against protein in lane 7. Lanes 3 to 6 were loaded with 12  $\mu$ g, lane 7 with 4  $\mu$ g, lane 8 with 25  $\mu$ g, and lane 9 with 10  $\mu$ g of protein. Dark bands on light background indicate Coomassie R-250 label in gels, and light bands on dark indicate western blotted proteins detected with anti-native aldolase B by ECL (Amersham); c) Elution profile for phosphocellulose chromatography, Relative Amounts: Protein,  $OD(280\text{ nm})$ ; Aldolase Activity, mU/10 (loaded then started washes when collecting fraction 9); FDP  $\downarrow$ , elution started with fructose 1,6-diphosphate.



**c.**

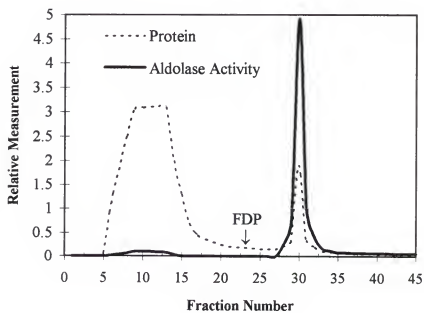


Figure 3-2: Antibodies Against Aldolase B. a) A preparative western blot of rat liver cytosol run on SDS-PAGE (see Coomassie in Fig. 3-6b, lane 8) was prepared and strips containing approximately 3  $\mu$ g total protein were probed with sera from rabbits immunized with native and denatured aldolase B as indicated, each lane contains a strip, numbers above each lane correspond to bleed numbers, and numbers with arrowheads indicate molecular weight. b) A Bio-Rad slot-blotting apparatus was used to load 10 ng/slot of antigen indicated by vertical labels (Aldolase A from Sigma), each row was probed with serum raised against antigen indicated by horizontal labels with increasing serum dilutions shown at the bottom of the figure, corresponding preimmune sera were used in rows immediately above and below anti-aldolase B and anti-Ub68, respectively.

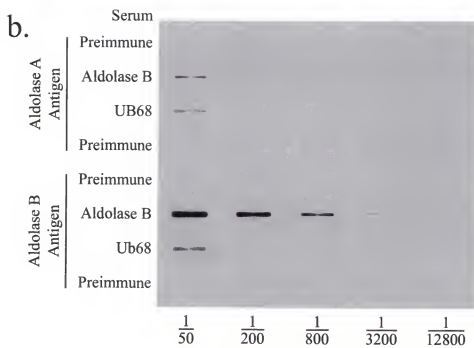
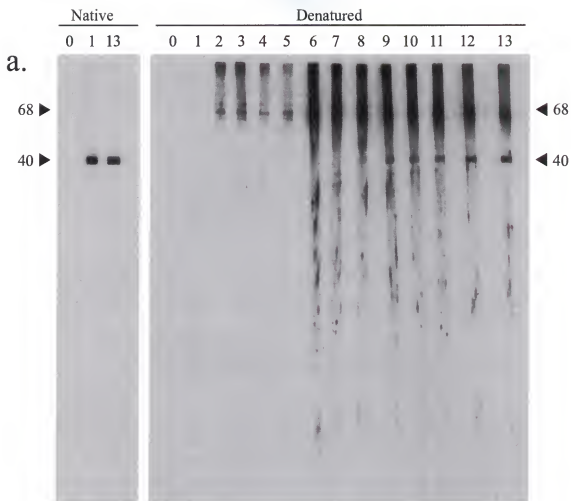
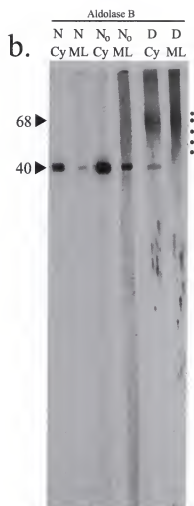
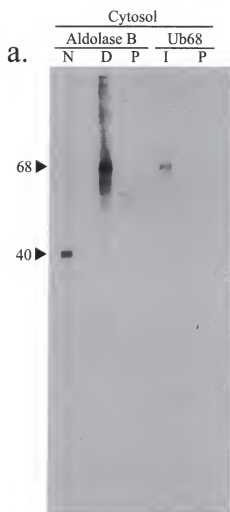


Figure 3-3: Aldolase B Ubiquitinated *In Vivo* is Enriched in Lysosomes. a) Preparative SDS-PAGE of cytosol from starved rat liver was western blotted onto nitrocellulose then cut into strips with ~3 µg protein/strip, and stained with early antisera against aldolase B (Fig.3-2a, bleed 4) or Ub68 (bleed 5); b) Aldolase B antisera from bleeds after booster injections (Fig.3-6a, bleed 10) were reacted with Cy (cytosol strips as prepared above) or ML (similar strips using a lysosome-enriched fraction instead). N, antiserum to active native enzyme; N<sub>o</sub>, same as N with 10-fold longer ECL exposure; D, antiserum to chemically denatured enzyme; I, antiserum to antigen extracted from polyacrylamide gel slices; and P, preimmune serum. Arrowheads, molecular weights in kD. Dots on rightmost edge indicate bands at molecular weights higher than expected for 40 kD aldolase B subunit. Susan E. Lenk provided subcellular fractions defined as follows: a 1,000 x g supernatant of rat liver homogenate was centrifuged at 6,000 x g; the resulting pellet was enriched in lysosomes and contained mitochondria (ML); the 6,000 x g supernatant was centrifuged at 100,000 x g yielding a supernatant fraction referred to as cytosol (Cy).



lanes u). Aldolase B was purified from the most productive strain, JM83, by cellulose phosphate chromatography (Fig. 3-1c) of a 45-60% ammonium sulfate cut (Fig. 3-1b, lane 5) from crude lysate (Fig. 3-1b, lane 3). Cellulose phosphate chromatography separates aldolase B by substrate affinity at the enzyme's active site, allowing enzymatically active aldolase B to be specifically eluted with fructose 1,6-diphosphate, FDP (Fig. 3-1c, peak at fraction #30). Aldolase B in peak fractions was at least 95% pure based on Coomassie stained SDS-PAGE gels (Figure 3-1b, lane 6) and specific activities ranging 0.95-0.98 U/mg (pure aldolase B = 1.0 U/mg). EDTA resistance of purified aldolase B activity indicated that contamination by class II bacterial aldolase was less than 0.005% (data not shown).

Aldolase B antigen described above was used to raise antibodies against native and chemically denatured aldolase B as detailed in Materials and Methods (Chapter 2). Preparative Western blots of rat liver cytosol were routinely used to follow specific immunoreactivities (Fig. 3-2a). Native antigen produced a highly reactive serum specific for 40 kD aldolase B. Anti-native aldolase B demonstrated minimal cross-reactivity with aldolase A (Fig. 3-2b). However, this antibody aldolase B effectively recognized aldolase B from different animal species (Fig. 3-2, a. 40 kD rat aldolase B, b. purified human aldolase B).

A rabbit immunized with denatured aldolase B produced antibodies (Fig. 3-2a "Denatured" bleeds 2 through 5) that specifically labeled a pattern indistinguishable from that for anti-Ub68 on western blots of subcellular fractions from rat liver (compare Fig.

3-3, lanes D with Figs 1-3 and 1-4, anti-Ub68). This demonstrated aldolase B epitopes in previously identified ubiquitin-protein conjugates and confirmed that aldolase B is ubiquitinated *in vivo*. The antibody to native aldolase B was specific for the 40 kD unmodified monomer. However, with ten-fold greater exposure times, even the anti-native aldolase B detected a ubiquitin ladder (Fig. 3-3b, N<sub>0</sub>). Preimmune sera failed to label any proteins. Antibodies raised against native aldolase B are known to be highly specific (Haimoto et al., 1989). Given this, labeling with anti-native aldolase B provides even stronger evidence that aldolase B is multiubiquitinated *in vivo*, and confirms that Ub68 is probably a stable conjugate of the form: (aldolase B)<sub>1</sub>(Ub)<sub>4</sub>.

#### Multiubiquitinated Aldolase B is Denatured and Enriched in Lysosomes

Aldolase A and B can spontaneously refold to active enzyme after reversible denaturation treatments (Horecker, et al., 1972; Beernink and Tolan, 1996). Anti-denatured aldolase B preferentially recognized ubiquitinated (> 40 kD) forms, whereas anti-native aldolase B preferentially recognized the unmodified (= 40 kD) form (Figs. 3-2a and 3-3). These results indicated that ubiquitination inhibits spontaneous refolding of aldolase B into native conformations. Reznick and Gershon also raised antibodies against native and denatured aldolase B (Reznick et al., 1985). Their anti-denatured aldolase B failed to immunoprecipitate catalytically active enzyme, but it effectively precipitated smaller peptides resulting from proteolysis. They also found that native aldolase B antibody failed to precipitate proteolytic fragments, but efficiently pelleted aldolase B activity (Reznick et al., 1985). The results reported in this study confirm the

hypothesis that native epitopes of aldolase B require three-dimensional conformations that mask denatured epitopes.

The 40 kD unmodified aldolase B predominantly occurred in cytosol (Cy) fractions with <10% in lysosome-enriched (ML) fractions (Fig. 3-3b). Consistent with previous results (Fig. 1-3), ubiquitin-aldolase B conjugates (bands >40 kD) were enriched in ML fractions with less occurring in Cy fractions (Fig. 3-3b). Taken together, the data suggest that ubiquitination can provide a mechanism for maintaining aldolase B in a denatured conformation. This could contribute to enhanced degradation of aldolase B by making degradative signals more accessible or by making the protein more vulnerable to proteases.

#### Heat Stress-Induced Delivery of Aldolase A to Lysosomes Requires Ubiquitination

Above, ubiquitinated aldolase B was confirmed to contribute to ubiquitin conjugates that are enriched in autophagic vacuoles and lysosomes during nutrient stress (Figs. 1-3, 1-4, and 3-3). The results suggested a role for ubiquitination in autophagic degradation of aldolase B. During heat stress, ubiquitin-dependent autophagic degradation of long-lived proteins has been demonstrated in E36 Chinese hamster lung cells (Gropper, et al., 1991; Handley-Gearhart, et al., 1994). Our results suggested that aldolase B was a possible substrate for this mechanism. However, E36 cells express endogenous aldolase A but not aldolase B (described later). Both aldolase A and B are established substrates for autophagy (reviewed in Chapter 1), so aldolase A was examined as a substrate for ubiquitin-mediated delivery to lysosomes during heat stress.

In addition to autophagy, temperature-dependent cytosolic proteolysis contributes to increased protein degradation during heat stress (next chapter; Hough and Rechsteiner, 1984). During autophagy, cytosolic proteins, like aldolase A, are sequestered into organelles (autophagic vacuoles and lysosomes) that can be pelleted by differential centrifugation. To measure effects specific for the autophagic pathway, aldolase A activity associated with pelletable organelles was assayed.

To examine a role for ubiquitination in the stress-induced degradation of aldolase A in lysosomes, a system developed by Schwartz and Ciechanover was utilized for measuring ubiquitin-dependent degradation of long-lived proteins (Gropper et al., 1991). During heat stress, E36 cells undergo enhanced autophagic degradation of long-lived proteins. However, ts20 cells derived from E36 cells harbor a temperature-sensitive mutation in ubiquitination. Heat stress is non-permissive for the mutation, so ubiquitination and enhanced autophagic degradation is inhibited in ts20 cells. The degradative phenotypes were confirmed and are presented at the end of this chapter.

FDP aldolase activity was used to follow endogenous aldolase A, and acid phosphatase activity was used to follow organelles in subcellular fractions collected by differential centrifugation as described in Materials and Methods (Fig. 3-4). Fractionation was optimized to maximize organelles released from cells, indicated by loss of acid phosphatase from low-speed centrifugation pellets (LP), and to maximize organellar integrity, indicated by fraction of acid phosphatase retained in high-speed centrifugation pellets (HP). Acid phosphatase occurs as both an integral membrane

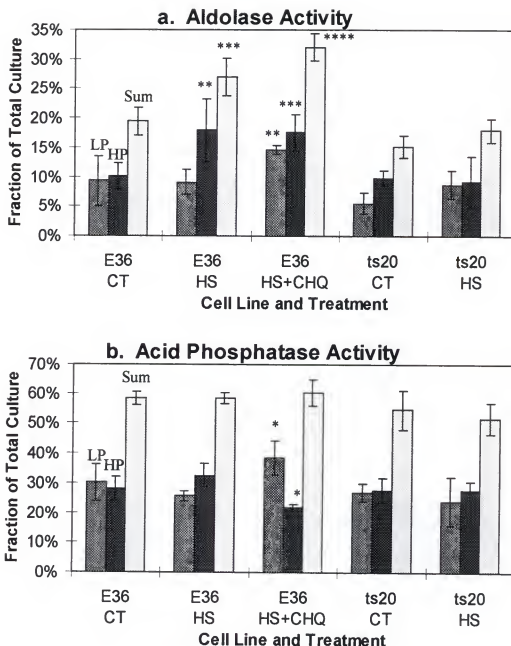


**Figure 3-4: Ubiquitin-Dependent Association of Endogenous Aldolase A with Organelles.** Aldolase (a.) and acid phosphatase (b.) reported as % total culture activity (mean  $\pm$  SD,  $n = 3$  cultures) for subcellular fractions collected from E36 and ts20 cells treated for 8.5 h as indicated; CT, control temperature; HS, heat stress; +CHQ, 80  $\mu$ M chloroquine; subcellular fractions were collected (Materials and Methods) and are labeled only on the first set of three bars (E36, CT): LP, low-speed pellet (1000x g); HP, high-speed pellet (100,000x g); Sum, total pelleted fractions (LP + HP); different from CT, Student's  $t$ -test: \*,  $p < 0.06$ ; \*\*,  $p < 0.03$ ; \*\*\*,  $p < 0.009$ ; \*\*\*\*,  $p < 0.0008$ .

protein and as a soluble matrix protein inside lysosomes. For organelles isolated here, acid phosphatase activity (about 60% of HP) was released by freeze-thaw (data not shown), indicating that much of it was soluble in E36 cell lysosomes and served as an adequate indicator of organellar integrity.

E36 cells were incubated at control (CT) and heat stress (HS) temperatures and subcellular fractions pelleted during differential centrifugation were characterized for aldolase and acid phosphatase enzymatic activities (Figs. 3-4 and 3-5). Relative to CT, HS treatment significantly increased aldolase activity distributed in pelletable fractions isolated from E36 cells (Fig. 3-4a). During 8.5 hour incubations that were used, partitioning of aldolase to pelletable compartments had to be faster than loss. This is consistent with accumulation of nascent autophagic vacuoles peaking by 6 hours after autophagic induction (Lawrence and Brown, 1992). Chloroquine (+CHQ) caused a more significant increase in pelletable aldolase activity. The effect of chloroquine suggests that lysosomal degradation contributes to aldolase A flux out of pelletable organelles consistent with an autophagic mechanism. In support of this, accumulation caused by heat stress and chloroquine (Fig. 3-5, HS+CHQ) corresponds to a  $1.5 \pm 0.3\%/h$  (mean  $\pm$  SD,  $n = 3$ ) increase in the sequestration rate for aldolase A which was similar in magnitude to induced autophagic degradation for total long-lived proteins (data presented in a later section). The results support endogenous aldolase A of E36 cells undergoing autophagic delivery to lysosomes during heat stress.

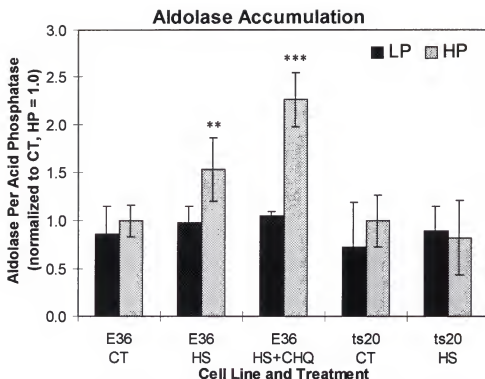


**Figure 3-5: Ubiquitination Mediates Lysosomal Accumulation of Aldolase A During Heat Stress.** Using enzyme activities collected for Figure 3-4, aldolase was divided by acid phosphatase to indicate relative aldolase associated with organelles (mean  $\pm$  SEM,  $n = 3$ ); values were normalized to the 100,000  $\times$  g pellet (HP) of control temperature (CT) to reflect aldolase accumulation relative to unstressed conditions. Labels are as in Figure 3-4. Student's t-test: \*\*,  $p < 0.03$ ; \*\*\*,  $p < 0.009$ .

Heat stress (HS) by itself failed to affect the distribution acid phosphatase activity in subcellular fractions (Fig. 3-4b). This indicated that increase of aldolase A in pellets was not due to redistribution of lysosomal organelles and supported the idea that the aldolase A was undergoing enhanced accumulation. Chloroquine treatment (+CHQ) had no effect on total pelletable acid phosphatase (Sum) but caused a redistribution of acid phosphatase from high-speed pellets (HP) to low-speed pellets (LP). It is known that

chloroquine treatment causes lysosomes to swell (Glaumann, et al., 1986). As a weak base, chloroquine accumulates in organelles proportional to their acidity, and mature lysosomes are the most acidic organelles. The results here indicate that some lysosomes became large enough to pellet at lower centrifugation speeds.

A basic result of subcellular fractionation is that different pelleted fractions have different contents of organelles (Rickwood, 1992). To demonstrate that aldolase A accumulates in a subpopulation of organelles (presumably lysosomes), aldolase activity was normalized to acid phosphatase activity and calculated the accumulation of aldolase A in HP and LP relative to lysosomal content (Fig. 3-5). Significant accumulation of aldolase activity only occurred in HP fractions during heat stress. In the presence of chloroquine (+CHQ), there was a greater than two-fold accumulation of aldolase activity in HP fractions but not LP fractions. The results suggest that aldolase A containing organelles were preferentially isolated in HP even during CHQ treatment, and are consistent with heat stress causing accumulation of aldolase A in a subpopulation of lysosomes.

A previous study has shown that the fractional volume of autophagic vacuoles and lysosomes does not significantly increase in heat stressed E36 cells (Lenk, et al., 1992). Together, the data indicate that heat stress increases the flux of aldolase A into autophagic vacuoles during heat stress. Unlike wildtype E36 cells, heat stress-induced accumulation of aldolase activity with pelletable organelles failed to occur for mutant ts20 cells (Figs. 3-4 and 3-5). Since heat stress inhibits ubiquitination in ts20 cells, this

suggested that aldolase A accumulation in organelles requires ubiquitination. The data support a role for ubiquitination in heat-stress induced sequestration of aldolase A.

Using electron microscopic morphometry, a previous study shows that in heat stressed ts20 cells conversion of autophagic lysosomes into residual bodies is specifically inhibited, resulting in a 6-fold accumulation of lysosomal volume (Lenk, et al., 1992).

The subcellular fractionation results here indicate that earlier events in autophagic degradation (aldolase A sequestration) might also involve ubiquitination. In conclusion, the endogenous aldolase A of E36 cells appears to require ubiquitination for heat-stress induced delivery to lysosomes.

#### Heat Stress-Induced Lysosomal Proteolysis of Aldolase B Requires Ubiquitination

Earlier in this chapter, ubiquitinated aldolase B in liver was shown to contribute to ubiquitin conjugates that are enriched in autophagic organelles during starvation-induced autophagy. Ubiquitination was required for heat stress-induced delivery of endogenous aldolase A to lysosomes of E36 cells, suggesting that aldolase A was degraded via ubiquitin-mediated autophagy. To examine whether aldolase B undergoes ubiquitin-mediated autophagy like aldolase A, subcellular fractionation studies in the last section were repeated with E36 and ts20 cells expressing epitope-tagged aldolase B (RABM).

E36 and ts20 cells were transfected and selected for permanent expression of rat aldolase B with the 9E10 myc epitope on its carboxyl terminus (RABM). The 9E10 epitope allowed efficient immunoprecipitation needed for degradation assays and

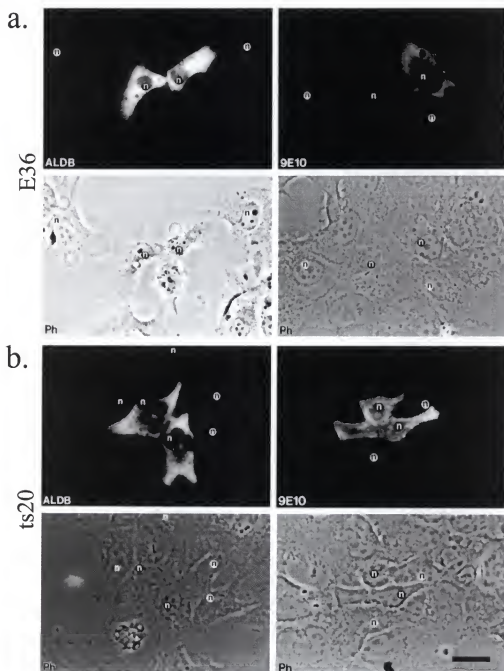


**Figure 3-6: Transient Expression of RABM.** a) E36 cells and b) ts20 cells transiently expressing rat aldolase B with a carboxyl terminal myc tag (RABM) were processed for immunofluorescence microscopy (Materials and Methods) and labeled with antibody against aldolase B (ALDB) or the myc epitope (9E10). Phase contrast (Ph) images for corresponding fields are shown below each immunofluorescent micrograph. Scale bar = 50  $\mu$ M.

unambiguous identification of the exogenous aldolase B, RABM. In Figure 3-6, transient expression of RABM was easily detected in a small fraction of cells with antibody to either the myc epitope (9E10) or to native aldolase B (ALDB). Many unlabeled cells indicated that E36 and ts20 cells do not express endogenous aldolase B. Cell lines were isolated and screened for permanent RABM expression, and the highest expressing lines for E36 and ts20 cells were designated E36AB and ts20AB, respectively (Figs. 3-7 and 3-8).

After clonal selection all cells in a microscopic field were labeled for RABM in permanent cell lines. Though most cells were brightly labeled, some were only dimly labeled. Such labeling remained constant after multiple culture passages and for different cell lines, suggesting that the variability was a trivial artifact of the immunofluorescence protocol. According to immunofluorescence and western blot assays, different cell lines had characteristic RABM levels that were maintained after multiple passages (data not shown). Control experiments performed with cell lines expressing 5 to 10 fold differences in RABM levels gave similar results. To facilitate detection, the highest expressing lines (E36AB and ts20AB) were used extensively, and data are reported for these lines. Immunofluorescent morphology indicated that RABM predominated in the cytosol as shown by the presence of negatively labeled nuclei and vacuoles, providing evidence that the recombinant protein demonstrated normal localization (Figs. 3-6, 3-7, and 3-8).

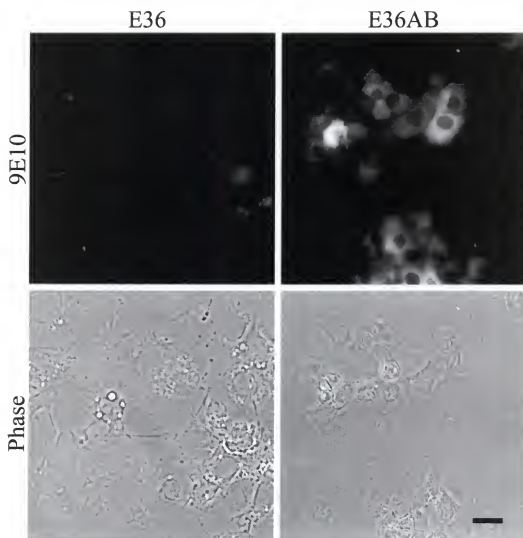


**Figure 3-7: Permanent Expression of RABM in E36 Cells.** Transiently transfected E36 cells were selected and screened for permanent RABM expression; the highest expressing cell line (E36AB) and untransfected cells (E36) were processed for immunofluorescent detection as described in Materials and Methods with anti-9E10 myc epitope (9E10, upper panels); phase contrast for corresponding fields are shown (Phase, lower panels) . Scale bar = 50  $\mu$ M.

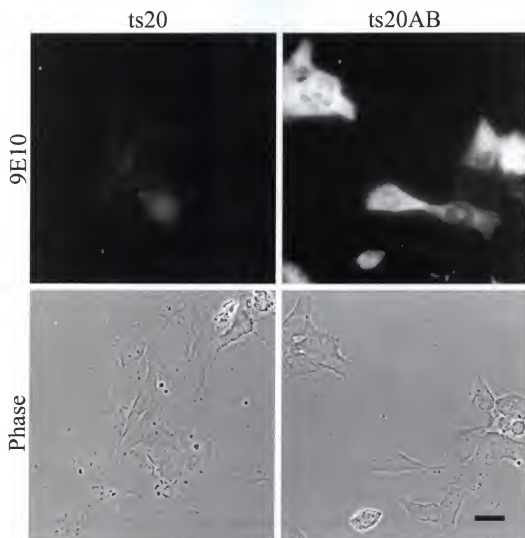


**Figure 3-8: Permanent Expression of RABM in ts20 Cells.** Transiently transfected ts20 cells were selected and screened for permanent RABM expression; the highest expressing cell line (ts20AB) and untransfected cells (ts20) were processed for immunofluorescent detection as described in Materials and Methods with anti-9E10 myc epitope (9E10, upper panels); phase contrast for corresponding fields are shown (Phase, lower panels) . Scale bar = 50  $\mu$ M.

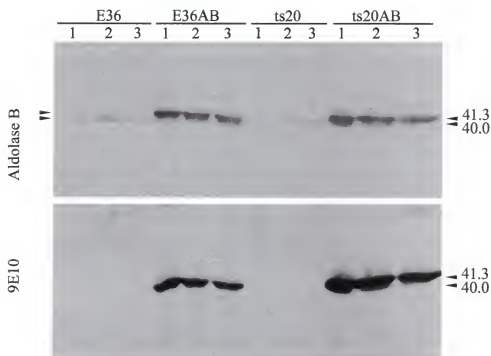


**Figure 3-9: Biochemical Detection of RABM Expression.** Three confluent cultures (1, 2, 3) for each of the indicated cell lines was trypsinized, pelleted, suspended in 2 x sample buffer, boiled, divided into two aliquots, run on duplicate SDS-PAGE gels, western blotted, and duplicated blots were stained for aldolase B (upper panel) or the 9E10 myc epitope (lower panel); E36, original Chinese hamster lung cell line; ts20, an E36-derived ts-mutant in ubiquitination; E36AB; an E36-derived cell line permanently expressing RABM; ts20AB, a ts20-derived cell line permanently expressing RABM. Arrow heads mark molecular weights (kD) for a doublet detected with anti-aldolase B.

On western blots of whole cells labeled with anti-aldolase B (Fig. 3-9), a single faint 40 kD band was detected in untransfected cells (E36 and ts20), consistent with cross-reactivity to aldolase A (Fig. 3-2b). Lysates of E36 and ts20 cells had aldolase cleavage activity 35 to 40 fold higher for fructose 1,6-diphosphate than for fructose-1-phosphate (data not shown). This difference for the two aldolase substrates is characteristic for aldolase A, identifying this isoform as the prevalent endogenous aldolase of E36 cells; note, aldolase B was not detected by immunofluorescence (Fig. 3-6).

For RABM-expressing cells (E36AB and ts20AB), a closely spaced doublet of bands was labeled with aldolase B antisera on western blots of whole cells (Fig. 3-9). One band occurred at 40 kD coincident with endogenous aldolase A of untransfected cells (E36 and ts20). A second strongly labeled band occurred at SDS-PAGE mobility corresponding to  $41.3 \pm 0.1$  kD (mean  $\pm$  SEM,  $n = 6$ ). On duplicate blots labeled for the C-terminal myc epitope of RABM (9E10), long luminographic exposure times only showed the 41.3 kD band (Fig. 3-9). 41.3 kD matched the predicted molecular weight of RABM, confirming that E36AB and ts20AB cells express full-length RABM.

If differences between immunoreactivities for hamster aldolase A (E36 cell endogenous) and rat aldolase B (RABM) are similar to differences between titrated immunoreactivities (Fig. 3-2b) for purified rabbit aldolase A (Sigma) and purified human aldolase B (Fig. 3-1), then immunoreactivities can be used to estimate RABM expression relative to endogenous aldolase A. Endogenous aldolase A immunoreactivity was below

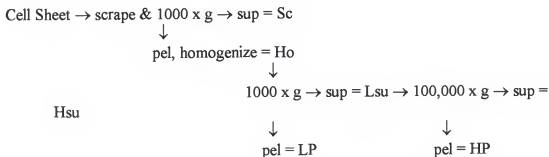
the linear range of western blot ECL assays in sample sizes subsaturating for aldolase B detection. Immunodetection in the low range variably underestimated aldolase A by 3 to >5 fold (data not shown), but allowed an upper limit for relative RABM expression to be estimated. Estimates varied in the range of 0.2-2.4 RABM per endogenous aldolase A. Though not precise, these overestimates indicated that RABM levels in permanent lines were equal or less than the endogenous.

To determine whether RABM undergoes heat stress-induced autophagy, cultures of E36AB and ts20AB were incubated at control (CT) and heat stress (HS) temperatures and fractionated as done for untransfected cells in the previous section titled, "Heat Stress-Induced Autophagy of Aldolase A Requires Ubiquitination." Subcellular distributions for activities of aldolase A and acid phosphatase were indistinguishable between parental and RABM-expressing cell lines. The enzymatic activity for aldolase A is ~10 fold more than aldolase B (Penhoet and Rutter, 1975). Given this, aldolase B enzymatic activity was too low to detect above endogenous aldolase expression in E36 cells. To overcome this problem, aldolase B immunoreactivity was followed on western blots of subcellular fractions (Fig. 3-10).

Figure 3-10a compares the distribution of immunoreactivities for RABM (Aldolase B) and an integral membrane protein of lysosomes (LAMP2b) in subcellular fractions isolated from E36AB cells incubated at control temperatures (CT). Details of the subcellular fractionation are described in Materials and Methods. When cell sheets were scraped from culture dishes and pelleted, some soluble proteins leaked out of the cells, as indicated by the presence of RABM (Aldolase B) in the supernatant (Sc, Fig. 3-

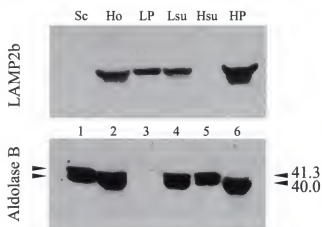
**Figure 3-10: RABM Associated With Lysosomes Undergoes Ubiquitin-Mediated Proteolysis.** Confluent E36AB and ts20AB cell cultures were incubated for 8.5 total hours at indicated conditions and then fractionated as described in Materials and Methods; samples of fractions were separated by SDS-PAGE, western blotted and labeled for distribution of lysosomal membranes on higher molecular weight half of blots (upper panels) and RABM on lower half of blots (lower panels) using antibodies to an integral membrane protein of lysosomes (LAMP2b) or to native aldolase B (Aldolase B); a) E36AB cells incubated in CT (see below) conditions were used to show distributions for lysosomal membranes and RABM which were comparable for all treatments within the variability of ECL detection (Amersham), 1% total culture equivalent of fractions was loaded per lane except HP (lane 6) which used 10%: Sc, supernatant from cells scraped then pelleted at 1000 x g; Ho, homogenate of lysed cell pellet; LP & Lsu, pellet & supernatant from 1000 x g of Ho; HP & Hsu, pellet & supernatant from 100,000 x g of Lsu; b) HP fractions isolated from cells incubated under indicated conditions were loaded for equal acid phosphatase activity to show relative content of full-length RABM (Aldolase B, 41.3), proteolyzed RABM (Aldolase B, 40.0), and lysosomal membranes (LAMP2b): Co, control temperature, in DMEM + 10% FBS (normal culturing conditions); CT, control temperature in MEM medium (experimental control); HS, heat stress in MEM medium; +CHQ, medium supplemented with 80  $\mu$ M chloroquine.

**Simplified Diagram of Fractions\*:**

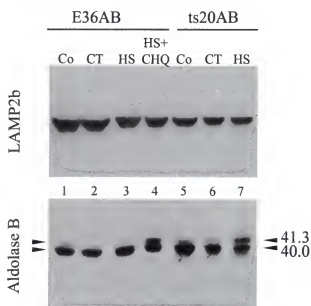


\*sup, supernatants; pel, pellets

## a. E36AB CT



## b. HP Fractions



10a, lane 1). Membrane-bound organelles were retained in cells as indicated by the lack of LAMP2b label in Sc.

The cell pellet was lysed, homogenized (Ho, Fig. 3-10a, lane 2), and used to produce 1000 x g (low speed) pellet (LP) and supernatant (Lsu). Low speed pellets generally contain nuclei, large cell fragments, and unbroken cells (Rickwood, 1992). LP contained no detectable RABM, indicating that most cells were broken enough to lose soluble proteins to Lsu (Fig. 3-10, lanes 3 and 4). Supporting this, LP contained about 15% of the aldolase A activity contained in Lsu (data not shown). The fact that some aldolase A (Fig. 3-4a) but no RABM was detected in LP is probably due to the greater affinity of aldolase A for pelletable cell components compared to aldolase B (Kusakabe, et al., 1997). LAMP2b was approximately equally distributed in LP and Lsu, indicating that about half the organelles (at least lysosomes) cofractionated with large cell fragments or nuclei. In agreement with this, acid phosphatase activity was similarly distributed between LP and Lsu (data not shown).

The Lsu was then used to produce 100,000 x g (high speed) pellet (HP) and supernatant (Hsu). Such high speed centrifugations pellet all membrane-bound organelles and leave soluble cytosolic (and leaked organellar) components in the supernatant (Rickwood, 1992). Consistent with this, all the detectable lysosomal membranes (Fig. 3-10a, LAMP2b) in Lsu (lane 4) were pelleted out of Hsu (lane 5). To make RABM (Aldolase B) labeling in HP (Figure 3-10a, lane 6) comparable to that loaded in lanes containing cytosol, 10 fold more HP equivalent was loaded.

On western blots of directly harvested whole cells (E36AB and ts20AB), aldolase B immunoreactivity (RABM) primarily occurred at 41.3 kD (Fig. 3-9). In subcellular fractions, a large proportion (>40%) of aldolase B immunoreactivity occurred at 40 kD (Fig. 3-10a). This indicated that 41.3 kD RABM was proteolyzed to ~40 kD size during fractionation. Like RABM, the LAMP2b-reactive protein was also proteolyzed as indicated by the presence of a smear below its band on western blots (Fig. 3-10a, especially visible in 10X loaded HP, lane 6). EDTA and storage on ice was used to reduce protein degradation in subcellular fractionations but was insufficient to prevent this presumably artifactual proteolysis. However, this result suggested that processing of RABM from 41.3 kD to 40 kD could be used as an indicator of proteolysis.

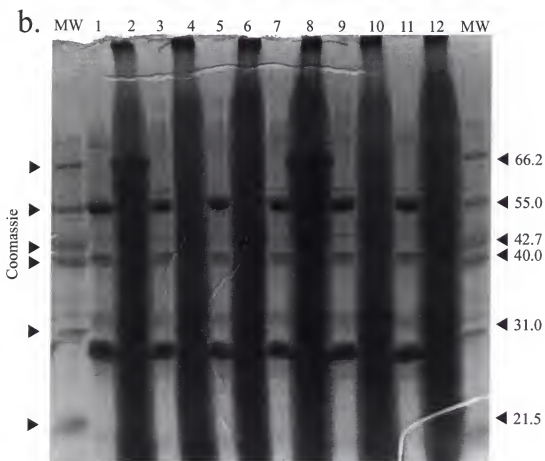
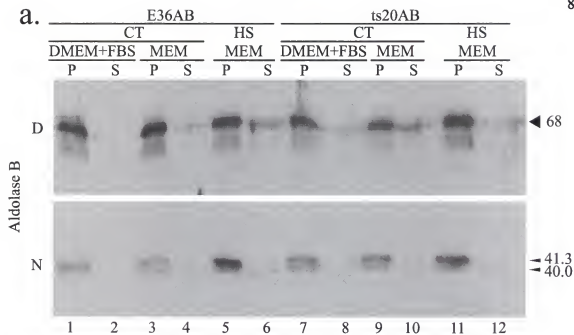
Consistent for all treatments and fractions containing RABM (except HP), the artifactual proteolysis was limited to processing 40-60% of the RABM (Fig. 3-10a, lanes 1, 2, 4, and 5). Only in HP fractions, processing of 41.3 kD RABM to 40 kD was complete or nearly complete, such that aldolase B immunoreactivity collapsed from a doublet to a single band at 40 kD (Fig. 3-10a, lane 6; Fig. 3-10b, lanes 1, 2, 3, 5, and 6). However, if lysosomal degradation was inhibited by chloroquine (E36AB, HS+CHQ) or autophagic degradation blocked by the non-permissive ubiquitination of heat stressed ts20 cells (ts20AB, HS) then the complete processing of RABM in HP fractions was blocked, as indicated by the persistence of 41.3 kD RABM in a doublet (Fig. 3-10b, lanes 4 and 7). These data indicated that RABM proteolysis occurred in lysosomes (CHQ-sensitive) and required ubiquitination (blocked in HS ts20AB). Whether greater

proteolysis of RABM seen in HP occurred in cells or during fractionation was not determined. With the data from the last section, the results suggest that RABM (aldolase B), like endogenous aldolase A, utilizes lysosomal degradation that requires ubiquitination in E36 cells.

To demonstrate the presence of ubiquitinated aldolase B in E36 cells expressing RABM, E36AB and ts20AB cell cultures were incubated at the different conditions used for subcellular fractionation studies above. Then RABM protein was isolated using 9E10-specific immunoprecipitation and separation on SDS-PAGE (Materials and Methods). Gels were western blotted and detected with native (N) and denatured (D) aldolase B antisera. Since antisera for denatured aldolase B were most sensitive and specific for ubiquitinated aldolase B on western blots (Fig. 3-2a and 3-3), these antibodies were used to probe for ubiquitinated aldolase B (Fig. 3-11a, upper panel). A major stable ubiquitin-aldolase B conjugate occurred at 68 kD consistent with Ub68 found in rat liver (Fig. 1-3 and 3-3) and in Fao hepatoma cells (Fig. 1-4). This confirmed that E36AB and ts20AB cells contained ubiquitinated aldolase B, including Ub68. Similar levels of Ub68 were detected in all conditions, including heat stressed ts20 cells (ts20AB, HS MEM) in which ubiquitination is inhibited. Unchanged ubiquitinated protein under conditions of inhibited ubiquitination appears contradictory. Though greatly inhibited, a low level of ubiquitination continues in heat stressed ts20 cells, but this low level is insufficient to mediate cellular processes (Hischberg and Marcus, 1982; Kulka, et al., 1988; Gropper, et al., 1991). The results here suggest that low levels of ubiquitination were sufficient to maintain multiubiquitinated intermediates of aldolase B

Figure 3-11: Ubiquitinated Aldolase B Occurs in E36 and ts20 Cells Expressing RABM.

Replicate sets of E36AB and ts20AB cultures were treated with indicated media (DMEM +FBS or MEM) and temperatures (CT or HS), harvested, and immunoprecipitated with 9E10 antibody; the immunoprecipitate was pelleted (P) from the lysate and proteins remaining in the supernatant were precipitated with trichloroacetic acid (S); P and S samples were boiled in 2 x sample buffer, split in equal aliquots, and duplicate gels run on SDS-PAGE; a) Western blots to detect RABM immunoreactivity (Aldolase B) were made from one gel and upper and lower portions of blots were immunodetected with anti-denatured (upper panel, D) and anti-native (lower panel, N) aldolase B, respectively; b) the duplicate gel was Coomassie stained, lane numbers at the top of the Coomassie gel correspond to identical samples in lanes numbered at the bottom of the blots in (a.); MW, molecular weight markers; molecular weights (kD) are indicated at right.



(Ub68 probably contains 4 ubiquitins), but were insufficient to mediate the proteolysis of RABM detected in HP fractions (Fig. 3-10b, lane 7). The occurrence of Ub68 in different samples from different cell types (Figs. 1-3, 1-4, and 3-11) supports it as a stable basal intermediate that probably requires more ubiquitination to facilitate proteolysis. Alternatively, heat stress-induced ubiquitination could operate on the machinery of stress-induced degradation. Further experiments are needed to distinguish these alternatives.

As detected with antiserum to native aldolase B (N), immunoprecipitation with the 9E10 epitope (specific for RABM) effectively pelleted all detectable 41.3 kD RABM protein and more than half of a dim-labeled 40 kD protein, presumably endogenous aldolase A (Fig. 3-11a, lower panel). The 40 kD aldolase A was shown to lack 9E10 immunoreactivity (Fig. 3-9), indicating that RABM and endogenous aldolase A occur as a complex in E36AB and ts20AB cells. This is consistent with the known tetrameric structure of all FDP aldolase isozymes, wherein different subunits randomly and stably oligomerize during synthesis (reviewed in Chapter 1). Ubiquitinated forms of aldolase B were also removed from lysates by 9E10 immunoprecipitation (Fig. 3-11a, upper panel), suggesting that ubiquitinated RABM retained its C-terminal epitope tag or retained associations with unmodified 9E10-immuno-reactive RABM subunits. If ubiquitinated RABM is not associated with other aldolase subunits, then this would provide evidence that ubiquitination might disassemble quaternary structure of aldolase B perhaps as an early step in the degradative pathway. However, this possibility was not pursued here.

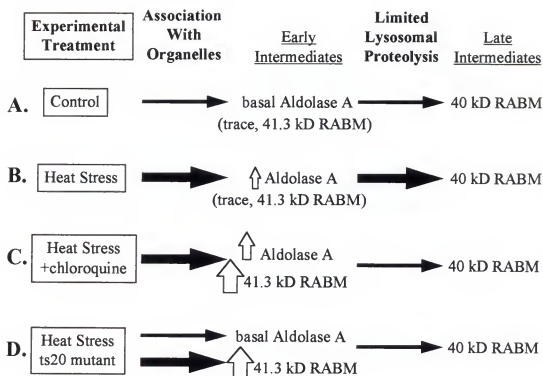


**Figure 3-12: Summary of Association and Limited Proteolysis of RABM and Endogenous Aldolase A in Pelleted Organelles of E36 Cells.** Each pathway corresponds to experimental treatment described in boxes at left and correspond to the following abbreviations used above: A) E36AB or ts20AB, CT; B) E36AB, HS; C) E36AB, HS + CHQ; D) ts20AB, HS. Since aldolase A was detected by enzymatic activity and is inactivated by limited proteolysis, no late intermediates of Aldolase A are shown. Since RABM detected on western blots shifts from 41.3 kD to 40 kD forms by limited proteolysis, these forms are listed for early and late intermediates, respectively. Weight of white vertical arrows indicate relative increases in detected levels of aldolase A and RABM pelleted with organelles. Weight of black horizontal arrows indicate relative rates proposed for processes listed in the heading.

Figure 3-12 summarizes the results of subcellular fractionation studies for endogenous aldolase A and RABM expressed in E36 cells. Aldolase A activity is very sensitive to proteolytic inactivation and loses 98% of its activity upon limited proteolysis (Penhoet and Rutter, 1975; Horecker, et al., 1985). Since this made proteolyzed

aldolase A undetectable in the background of active aldolase A from E36 cells, aldolase A activity was used to demonstrate association with organelles but not for detecting proteolyzed intermediates of degradation. RABM was detected by western blotting with antiserum to native aldolase B, allowing detection of limited proteolysis (41.3 kD  $\rightarrow$  40 kD) products referred to here as "late intermediates" (Fig. 3-12, last column). Smaller molecular weight intermediates of proteolysis were not detected, because they are not recognized by anti-native aldolase B and probably are more rapidly degraded than 40 kD aldolase B (Reznick, et al., 1985; Horecker, et al., 1985).

Under control conditions (Fig. 3-12, A), basal levels of aldolase A, 40 kD RABM, and a trace of 41.3 kD RABM were detected in pelletable organelles. Heat stress (Fig. 3-12, B) caused a partial increase in aldolase A activity with little apparent change in RABM forms. However, a partial increase in trace levels of 41.3 kD RABM were likely to be missed, because they were below the threshold for optimal Enhanced Chemiluminescent detection (Amersham). Consistent with reaching the threshold for detection, chloroquine inhibition of limited proteolysis caused a sudden signal increase in 41.3 kD RABM (Fig. 3-12, C). Chloroquine also caused an even more aldolase A to accumulate. The results indicated that lysosomal proteolysis mediates loss of aldolase A and aldolase B (RABM) associated with organelles, demonstrating sequestration of these proteins into lysosomes.

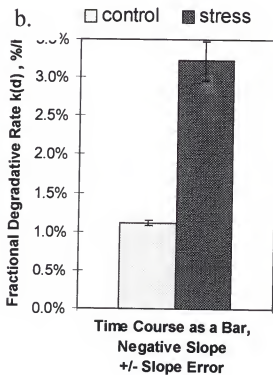
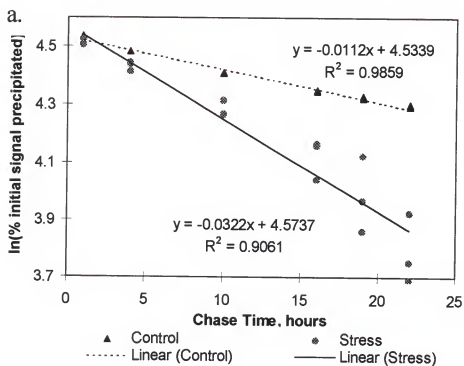
When ubiquitination was inhibited by the ts20 mutation (Fig. 3-12, D) different results were obtained for aldolase A and aldolase B (RABM). Consistent with

ubiquitination mediating degradative mechanisms after sequestration (Lenk, et al., 1992), 41.3 kD RABM accumulated in lysosomes. Under the same conditions, aldolase A activity did not accumulate. For this to happen, reduced ubiquitination in ts20 cells would have to increase lysosomal proteolysis or decrease sequestration of aldolase A into lysosomes. Since the former possibility is unlikely, aldolase A probably requires ubiquitination for sequestration as well as proteolysis. Since assays for aldolase A and RABM were fundamentally different, the difference in ubiquitination effects between the two isoforms might be due to differences in detection by enzymatic activity versus immunoreactivity. Further experiments are needed to address this problem, but the results here suggest that both aldolase A and aldolase B require ubiquitination for degradation in lysosomes.

#### Ubiquitin-Mediated Autophagic Degradation Occurs in E36AB Cells

Above, evidence supporting ubiquitin-mediated proteolysis of aldolase B (RABM) in lysosomes during heat stress was presented. To confirm that ubiquitin-mediated autophagic degradation occurred during the fractionation studies above, degradation rates of total long-lived proteins were measured for E36AB and ts20AB cells under the same conditions. All protein degradation rates reported in this dissertation are calculated as for degradative rate constants. Procedures are described in Figure 3-13 and Materials and Methods. Arbitrary examples of regression analysis for basal (control) and induced (stress) degradation rates are indicated in Figure 3-13. The degradative rate constant,  $k_d$ , is defined as the negative slope (coefficient of  $x$ ) of the

Figure 3-13: Quantification of Degradation Rates. a) Cells were metabolically radiolabeled and chased as described in Materials and Methods. Radiolabel signal (less background) in TCA precipitates (for total long-lived proteins) or at appropriate molecular weights for immunoprecipitates run on SDS-PAGE (for aldolase B) was measured and magnitudes were natural-log transformed and plotted as described in Materials and Methods. Regression lines, equations, and  $R^2$  correlation statistics are shown for arbitrary examples of basal (Control) and heat stressed (Stress) protein degradation. b) Slopes reported as decimal numbers in (a) are plotted as bars and reported with units of %/h ( $= \text{kd} / \text{h} \times 100\%$ ). The SEM of the slope was calculated using Microsoft Excel Spreadsheet Analysis package: ANOVA regression error (standard error of y-values at given x-values divided by the square root of the deviations squared of x-values).



lines plotted in Figure 3-13a and of signal decrease in this dissertation are calculated as done for  $k_d$ . Note of caution: different methods of calculating degradation are used by different investigators which would give different rate magnitudes for the same data; rates presented here are based on the empirically supported assumption that protein degradation mechanisms operate with first order kinetics.

In this chapter, degradation rates are presented for total trichloroacetic acid (TCA) precipitated proteins. Such rates represent an average of the pooled degradative rate constants ( $k_d$ ) for all radiolabeled proteins over the time interval of the assay. Variation in metabolic radiolabeling and chase intervals yield differentially labeled total proteins due to widely different turnover rates for individual proteins, and such variation would change the pooled  $k_d$ . These factors were controlled to give comparable rates between different experiments throughout this dissertation.

To confirmed that E36 cells undergo heat stress enhanced degradation of total protein that requires ubiquitination, E36 cells, ts20 cells, and ts20E1 cells were radiolabeled then chased under control (CT) and heat stress (HS) conditions, and degradation rates were calculated as described above (Fig. 3-13). E36 cells were found to undergo about a two fold enhancement in the degradation of long-lived proteins (Fig. 3-14, E36). The ts20 cell line derived from E36 cells has a temperature-sensitive mutation in E1 ubiquitin-activating enzyme, and the heat stress (HS) is non-permissive for E1 function in ts20 cells, greatly inhibiting ubiquitination (Kulka, et al., 1988) and ubiquitin-mediated protein degradation (Gropper, et al., 1991). Consistent with this, the

reported as the fractional degradative rate in Figure 3-13b. For consistency and to allow direct comparisons of data, all rates HS-enhanced degradation of long-lived proteins was blocked in ts20 cells (Fig. 3-14, ts20). Furthermore, the mutant degradative phenotype was rescued in ts20 cells permanently expressing wild type human E1 (Fig. 3-14, ts20E1).

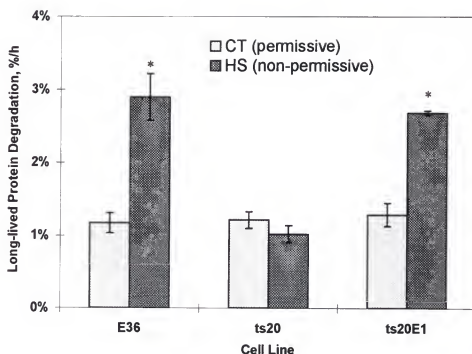


**Figure 3-14: Heat Stress-Induced Degradation of Long-lived Proteins in E36 Cells Requires E1-Mediated Ubiquitination.** Cultures of parent (E36) cells, E1-ubiquitination mutant (ts20) cells, and mutant rescued with cDNA for wildtype human E1 (ts20E1) cells were assayed for degradation of total TCA precipitated long-lived proteins radiolabeled with  $^{14}\text{C}$ -valine (Fig. 3-13, Materials and Methods) reported for the indicated conditions (mean  $\pm$  SEM,  $n = 9$ ); CT, control temperature; HS, heat stress; \*, HS > CT (Student's  $t$ -test,  $p < 0.000001$ ).

The results demonstrate that heat stress-induced degradation in E36 cells is dependent on ubiquitination mediated by E1. The degradation of long-lived proteins in E36AB and ts20AB cells was assayed (Fig. 3-15), and the results were similar to those for untransfected E36 and ts20 cells (Fig. 3-14). This demonstrated that RABM expression had no effect on the degradative phenotypes in these cells. Cultures were also treated with the relatively specific inhibitor of autophagy, 3-methyladenine (3MA), or with the lysosomal hydrolase inhibitor, chloroquine (CHQ). Both inhibitors effectively blocked the enhanced degradation of long-lived proteins (Fig. 3-15). The results confirmed that heat stress-induced degradation of most long-lived proteins occurred in lysosomes via autophagy.

In Figure 3-15, ts20AB cells appear to have reduced basal degradation relative to E36AB cells. This result was not consistent between experiments, and in general, E36 and ts20 cells had similar basal degradation rates that were unaffected by RABM expression (data not shown). Chloroquine (CHQ) and 3-methyladenine (3MA) inhibited a fraction of basal protein degradation at the concentrations shown (Fig. 3-15). Though the effect of chloroquine was marginal here (40  $\mu$ M), at higher concentrations (80 & 160  $\mu$ M) inhibition of basal degradation was more significant (data not shown). CHQ at the higher concentrations and 3MA at the least inhibitory concentration reduced the viability of ts20 cells during heat stress (Materials and Methods; data not shown), so degradation measurements for such treatments were not included. As indicated by sensitivity to CHQ or 3MA,  $17 \pm 6\%$  of basal degradation of long-lived proteins occurred by lysosomal degradation via autophagy, corresponding to a rate of  $0.3 \pm 0.1\%/h$  (mean  $\pm$  SEM,  $n =$

- 12). Given the estimated basal rate of autophagy, heat stress (HS) caused a calculated  $6.6 \pm 1.0$  fold induction over basal autophagic degradation in uninhibited wildtype E36

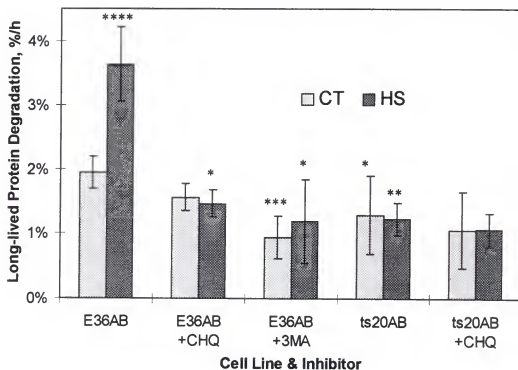


**Figure 3-15: Heat Stress-Induced Autophagic Degradation of Long-lived Proteins that Requires Ubiquitination Occurs in Cells Expressing RABM.** Cultures of E36AB and ts20AB cells were metabolically labeled with  $^{35}$ S-methionine, and at various times of chase, cultures were lysed in modified RIPA buffer and 2% of the lysate was TCA precipitated and processed to measure degradation of long-lived proteins (mean  $\pm$  SEM,  $n = 16-34$ ) as previously described (Fig. 3-13 and Materials and Methods); CT, control temperature; HS, heat stress; indicated chase medium were supplemented with 40  $\mu$ M chloroquine (+CHQ) or 5 mM 3-methyladenine (+3MA). Significant differences (Student's t-test,  $p < 0.05$ ): mean  $<$  untreated CT in corresponding cells (\*,  $p < 0.05$ ; \*\*,  $p < 0.02$ ; \*\*\*,  $p < 0.008$ ); \*\*\*\*, E36AB, HS  $>$  CT ( $p < 0.0002$ ). The remaining lysates (98% of the volume) were used to immunoprecipitate and measure the proteolysis of RABM discussed below (Fig. 4-4).

cells (mean  $\pm$  SEM,  $n = 3$ ). In ubiquitination mutant ts20 cells, enhanced degradation was blocked (Figs. 3-14 and 3-15; Gropper, et al., 1991), and a 6-fold increase in fractional volume of autophagic vacuoles occurred (Lenk, et al., 1992). Together, the data support a model in which intralysosomal complete degradation of proteins requires ubiquitination, but sequestration does not (Lenk, et al., 1992).

Basal autophagic degradation explained why aldolase A and RABM were found with pelletable organelles in all conditions (Fig. 3-4a and 3-10). Neither aldolase A activity nor RABM immunoreactivity accumulated in HS ts20 cells, even though autophagic sequestration was increased 6-fold (previous paragraph). Ubiquitination was required for limited proteolysis of RABM from 41.3 kD to 40 kD (Fig. 3-10b), but other limited proteolysis appeared sufficient enough to destroy enzyme activity and immunoreactivity, preventing multifold accumulation of detectable aldolase. Though detectable aldolase fragments did not accumulate, proteolysis did not seem to proceed to amino acids, because enhanced lysosomal degradation of TCA-precipitable polypeptides was blocked in HS ts20 cells (Figs. 3-14 and 3-15). The data are consistent with ubiquitination being required for a subset of lysosomal protease activities needed to completely degrade long-lived proteins to amino acids, and one such protease can be detected as intralysosomal limited proteolysis of RABM (aldolase B).

## CHAPTER 4: TEMPERATURE MODULATES AUTOPAGY AND CYTOSOLIC PROTEOLYSIS OF ALDOLASE B

### Introduction

In the literature, starvation-induced autophagic degradation of aldolase B has been proposed, and heat stress-induced autophagic degradation of long-lived proteins in E36 cells was shown to require ubiquitination. As shown above, ubiquitinated aldolase B (Ub68) occurred in cultured E36 cells and *in vivo* enriched with organelles of starvation-induced autophagy isolated from rat liver. In E36 cells, assays for aldolase associated with organelles showed that lysosomal accumulation of endogenous aldolase A and lysosomal proteolysis of exogenous aldolase B (RABM) required ubiquitination. The results are consistent with degradation of aldolase isoforms in general occurring by ubiquitin-mediated autophagic degradation inside lysosomes. However, aldolase A and aldolase B have been proposed to undergo limited proteolysis on the outside of lysosomes independent of acidic pH (Pontremoli, et al., 1982; Horecker, et al., 1985; Sygush, et al., 1990). To examine the contribution of cytosolic and lysosomal mechanisms, rates for basal and heat stress-induced proteolysis of aldolase B (RABM) in E36 cells were quantified in the presence of lysosomal inhibition. The results below show that temperature-dependent cytosolic proteolysis of aldolase B was independent of ubiquitination, that complete cytosolic protein degradation to amino acids was

independent of temperature, and that autophagic degradation also increased in a temperature-dependent manner.

#### Ubiquitin-Independent Cytosolic Proteolysis of Aldolase B

To measure proteolysis of aldolase B, the decrease of radioactivity in RABM protein bands on gels of 9E10-immunoprecipitates isolated from metabolically labeled cells during pulse-chase experiments was measured. Immunoprecipitation was efficient with anti-9E10 (Fig. 3-11a, lower panel, N) and consistently yielded more RABM than for anti-aldolase B (Fig. 4-1). 9E10 immunoprecipitation gave the most reproducible data and was used extensively for experimental repetitions; unless otherwise stated, degradation rates for RABM are reported for such data. Standard curves were done to confirm a linear response for quantification of the radiolabeled RABM (Fig. 4-2). Degradation rates were calculated in the same manner as for total long-lived protein degradation using RABM-specific signal (Fig. 3-13). Since a specific protein was measured, the resulting degradation rates were equivalent to the degradative rate constant ( $k_d$ ) for RABM (aldolase B) proteolysis. The results below agree well with published  $k_d$  for aldolase B degradation in cultured cells (1.0-2.5%/h basal and 3.0-5.0%/h stress-induced).

To determine the contribution of ubiquitination in heat stress-induced degradation of aldolase B, degradation rates for RABM in E36AB and ts20AB cells at control(CT) and heat stress (HS) temperatures were measured (Fig. 4-3). The

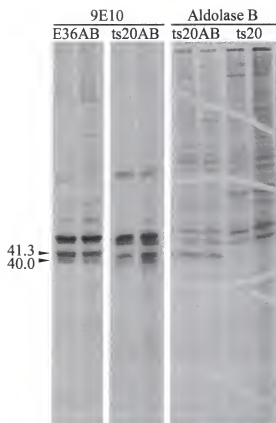


**Figure 4-1: Immunoprecipitation of RABM With Antibodies Against 9E10 or Native Aldolase B Epitopes.** Anti-myc-sepharose in mRIPA buffer lysates (9E10) or aldolase B antiserum precipitated with protein A-agarose in SLB lysates (Aldolase B) were used to immunoprecipitate radiolabeled RABM from E36AB and ts20AB cell lines; ts20 cells lacking RABM demonstrate background for the least stringent immunoprecipitation (Aldolase B). Arrowheads indicate only specifically precipitated bands (40 and 41.3 kD), migrating in a region of consistently low background. The prominent 44-45 kD band in 9E10 immunoprecipitations was non-specific, representing actin that has a high affinity for sepharose (Sigma technical support).

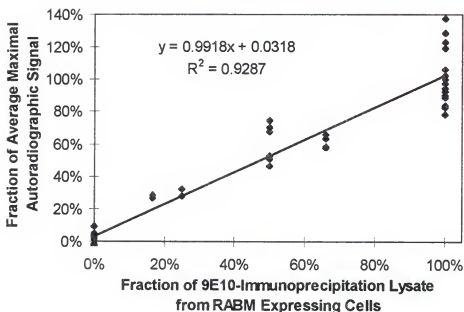


**Figure 4-2: Standard Curve For 9E10 Immunoprecipitation of RABM.** Confluent cultures of ts20AB and ts20 (untransfected) cells were metabolically labeled with  $^{35}\text{S}$ -methionine, lysed in mRIPA buffer, lysates precleared, and immunoprecipitated (anti-9E10 conjugated to sepharose) done as described in Materials and Methods, except ts20AB lysate (containing maximal RABM) was diluted with ts20 lysate (lacking RABM) as indicated. Each immunoprecipitation was done from ~400,000 cell equivalents of lysate. Regression line and equation for least squared deviation is shown with  $R^2$  correlation statistic;  $R = 0.9637$  (ideal would be a slope of 1.00 with intercept of 0.00 and  $R^2=1.00$ ).

degradation of RABM was two fold enhanced by heat stress. Though starvation-induced degradation of aldolase B is established (reviewed in Chapter 1), this is the first demonstration that aldolase B undergoes enhanced degradation during heat stress. Furthermore, induced proteolysis continued even when ubiquitination was inhibited (Fig. 4-3 HS,ts20AB), indicating involvement a ubiquitin- independent mechanism. This suggested that in addition to ubiquitin-mediated lysosomal proteolysis (Fig. 3-12), a

second proteolytic mechanism for RABM occurred at rates similar to heat stress-induced autophagy that required ubiquitination (Fig. 3-15).

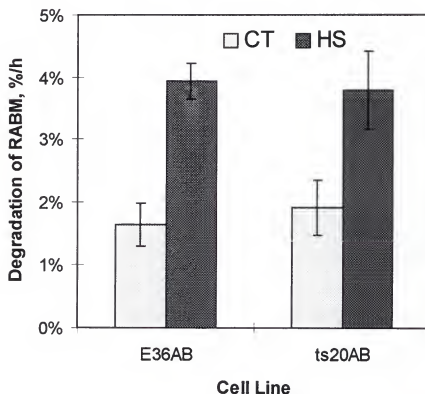


**Figure 4-3: Heat Stress-Induced Degradation of RABM Occurs Independently of E1-Mediated Ubiquitination.** Indicated cell lines were metabolically labeled with  $^{35}\text{S}$ -methionine, chased for various times, immunoprecipitated, radioactivity at the molecular weight of RABM was quantified, and regression analysis performed to calculate the degradative rate constant  $\pm$  standard error ( $n = 31$  to  $34$ ) for RABM as described in Materials and Methods, under control (CT) and heat stress (HS) conditions. Reminder: HS is non-permissive for E1-mediated ubiquitination in ts20AB cells. For both cell lines, RABM degradation at HS was higher than at CT ( $p < 0.0007$ ).

### Temperature-Dependent Cytosolic Proteolysis in Fao Cells

To determine how much heat stress-enhanced proteolysis of RABM occurred in lysosomes, chloroquine (CHQ) was used to inhibit lysosomal hydrolases (Fig. 4-4).

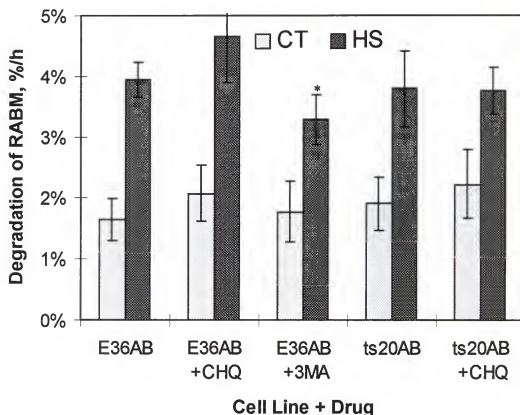


**Figure 4-4: Heat Stress-Induced Degradation of RABM Occurs Independently of Lysosomal Hydrolases and Autophagy.** Cultures were treated and processed as for Figure 4-18 to calculate degradation rates (mean  $\pm$  SEM,  $n = 16$  to  $34$ ), except cultures were treated with either  $5$  mM  $3$ -methyladenine ( $+3MA$ ) or  $40$   $\mu$ M chloroquine ( $+CHQ$ ) to inhibit autophagy or lysosomal hydrolases, respectively. Student  $t$ -tests: for all treatments,  $HS > CT$  ( $p < 0.0013$ ); \*,  $3MA$  partially inhibited  $HS$  degradation ( $p < 0.031$ ); all other differences were not significant at a  $0.05$  level. Note:  $2\%$  of each culture lysate was TCA precipitated and used to measure degradation of long-lived proteins occurring under identical conditions (Fig. 3-15).

Chloroquine failed to reduce the heat stress-induced degradation rate for RABM; note, the apparent increase shown in Fig. 4-4 was neither statistically significant nor reproducible (data not shown). Even when ubiquitination and lysosomal degradation were simultaneously inhibited (ts20AB+CHQ), enhanced degradation of RABM still continued (Figs. 4-4). This indicated that heat stress-induced proteolysis of aldolase B in E36 cells was independent of ubiquitin-mediated autophagy and lysosomal degradation. Supporting this, 3-methyladenine only marginally inhibited heat stress-induced autophagic degradation (Fig. 4-4), and the marginal inhibition was accounted by a comparable reduction of basal protein degradation observed for samples from the same cultures (Fig. 3-15). These results support a role for cytosolic proteolysis in the heat stress-induced degradation of aldolase B.

The inhibitor resistant proteolysis of RABM (Fig. 4-4) seemed to contradict the inhibitor sensitive degradation of total long-lived proteins measured for the same cultures (Fig. 3-15), suggesting that degradation of RABM occurred by a selective cytosolic mechanism which was specific for a fraction of total long-lived proteins that includes aldolase B. An alternative explanation is possible, because protein degradation measurements using TCA and immunoprecipitation are fundamentally different from each other. Degradation measurements for total proteins utilize TCA precipitation which cannot distinguish full length protein from polypeptide fragments created by partial proteolysis. However, immunoprecipitation is sensitive to limited proteolysis, because a single proteolytic cleavage can disrupt the size and structure of a protein

removing it from an SDS-PAGE band used to quantify degradation. Furthermore, aldolase B is known to undergo proteolysis to relatively large polypeptide fragments that cannot be recognized by antibodies against the native protein (Reznick, et al., 1985), demonstrating that partial proteolysis can disrupt immunoreactivity. Immunoreactivity lost to partial proteolysis causes loss of radiolabel from corresponding immunoprecipitates, including fragments that might be quite large. Thus, immunoprecipitation effectively detects limited proteolysis whereas TCA precipitation requires complete degradation to amino acids. Given this, cytosolic proteolysis of aldolase B (RABM) occurred independently by partial proteolysis that produced undetectable polypeptide fragments (TCA precipitable) requiring autophagic degradation in lysosomes for complete proteolysis.

Since polypeptide fragments of aldolase B could not be detected the possibility remains that aldolase B underwent an enhanced complete degradation in cytosol during heat stress. However, this would have to be a unique pathway for aldolase B because for most proteins (TCA precipitated) autophagic degradation was required. Given this, a cytosolic mechanism for complete degradation of aldolase B would have to be selective for aldolase B, requiring a recognition mechanism that is novel and unsubstantiated in the literature. This seems less likely than a more general model in which many proteins, including aldolase B, undergo partial cytosolic proteolysis followed by autophagic degradation in lysosomes.

Degradation assays using anti-aldolase B immunoprecipitation gave similar results to anti-9E10 (data not shown). This indicated that proteolysis of RABM was not limited to the epitope tag but included at least some degradation of aldolase B protein. Since immunoprecipitations were done from extracts of cell sheets, loss of RABM to the media would look like degradation. To control for this, heat stress release of aldolase activity into media was measured and found statistically insignificant compared to variation in induced degradation measurements (Fig. 4-5).

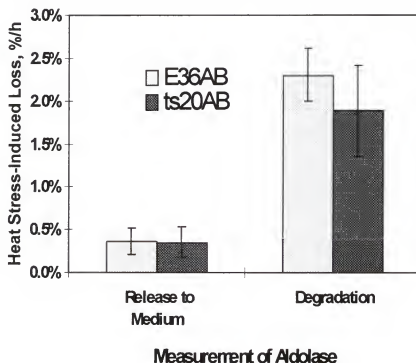


**Figure 4-5: Heat Stress-Induced Degradation of Aldolase B was Not Due to Release of Aldolase to Medium.** During chase treatments, aliquots of media were assayed for aldolase activity; after the final chase, cells were harvested, total activity in the culture was calculated, and the fractional rate of activity loss calculated. The rate calculation and measurements for the degradation of aldolase B are described in Materials and Methods. Release of aldolase to media  $> 0$  ( $p < 0.034$ ) but had a low probability of contributing an effect on degradation rates (Student's t-test,  $p < 0.0001$ ). At control temperatures (data not shown), aldolase activity released to the medium was not significantly greater than zero ( $p > 0.4$ ).

### Starvation-Induced Autophagic Degradation of Aldolase B in Fao Cells

To control for the nature of the epitope tag on RABM and determine whether cytosolic proteolysis of aldolase B generally occurred in different cells, heat stress-induced proteolysis of epitope-tagged human aldolase B (HAHAB) expressed in Fao cells was examined (next section). HAHAB carried a 12CA5 HA (hemagglutinin) epitope on its amino terminus. Measuring degradation of HAHAB in Fao cells versus RABM in E36 cells controlled for cell type (rat hepatoma vs. Chinese hamster lung), position and identity of the epitope tag (amino-HA vs. carboxyl-myc), and the source species of the recombinant aldolase B (human vs. rat). Whether heat stress can induce autophagy in Fao cells was not known. Before examining this possibility, it was reasonable to confirm that starvation-induced autophagic degradation of exogenous aldolase B (HAHAB) occurred like that for endogenous aldolase B.

Fao cells retain many characteristics of differentiated hepatocytes, including expression of endogenous aldolase B (Deschatrette et al., 1979; Deschatrette and Weiss, 1974), and starvation-induced degradation of long-lived proteins (Fig. 4-6, bar 2). Treatment of Fao cultures with 3-methyladnine (3MA) to block autophagic sequestration, chloroquine (CHQ) to inhibit lysosomal acid hydrolases, or leupeptin (LEUP) to inhibit lysosomal serine proteinases blocked starvation-induced degradation (Fig. 4-6), demonstrating that enhanced degradation of long-lived proteins occurred in lysosomes via autophagy in starved Fao cells as well as in heat stressed E36 cells. E36 cells did not undergo starvation-induced degradation (data not shown), but Fao cells

provided an opportunity to examine both starvation-induced and heat stress-induced degradation of aldolase B in the same cell type.

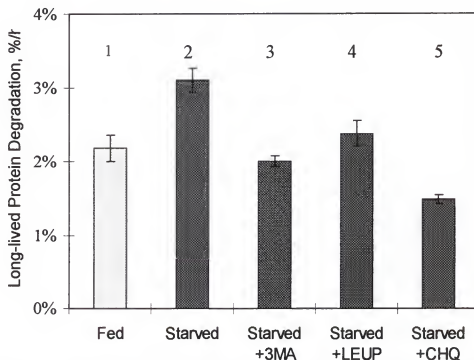


**Figure 4-6: Starvation Induces Degradation of Long-lived Proteins in Fao Cells.** Fao cell cultures were metabolically labeled with  $^{14}\text{C}$ -valine, chased with DMEM + 10% FBS (Fed, bar 1) or Krebs-Heinseleit Medium (Starved, bars 2-5) containing “cold” 10 mM valine, and loss of TCA precipitable counts was used to calculate degradation rates (mean  $\pm$  SEM,  $n = 8-16$ ) as described in Materials and Methods; Krebs-Heinseleit is an amino acid free minimal medium; 3MA (bar 3), 10 mM 3-methyladenine; LEUP (bar 4), 300 mM leupeptin; CHQ (bar 5), 160  $\mu\text{M}$  chloroquine. Significant differences (Student's  $t$ -test,  $p < 0.0001$ ): bar 2 > bars 1, 3, 4, or 5; bar 5 < bar 1.

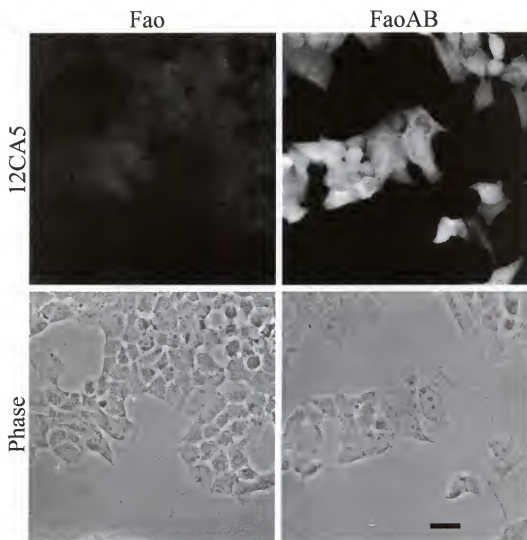


**Figure 4-7: Permanent Expression of HAHAB in Fao Cells.** Fao cells were transfected and selected for permanent expression of human aldolase B carrying a 12CA5 HA epitope tag on its amino terminus (HAHAB), resulting a cell line permanently expressing HAHAB (FaoAB). Untransfected Fao (Fao) and FaoAB cells were processed for 12CA5 immunofluorescence microscopy (upper panels) as described in Materials and Methods; Phase (lower panels), phase contrast of same field as upper panels. Scale bar = 50  $\mu$ M.

To effectively measure the degradation of aldolase B in Fao cells, an Fao cell line permanently expressing epitope-tagged aldolase B (HAHAB) was isolated. Figure 4-7 shows the immunofluorescent signal for the HA epitope (12CA5) in a cell line permanently expressing HAHAB (FaoAB). Note the bright signal in FaoAB cells relative to the untransfected parent cells (Fao). Anti-HA epitope (12CA5) specifically detected expression of tagged exogenous aldolase B as indicated by lack of label in untransfected cells (Fig. 4-7, Fao). The presence of negatively labeled nuclei and vacuoles supported a cytosolic localization for HAHAB. Consistent with Fao cells expressing endogenous aldolase B, Fao and FaoAB cells stained with anti-aldolase B had similar labeling patterns to each other (data not shown) which resembled the transfected cells labeled with anti-12CA5 (Fig. 4-7, FaoAB).

Expression of HAHAB was confirmed on western blots (Fig. 4-8). Western blot analysis with anti-aldolase B (Fig. 4-8a) showed 40 kD endogenous aldolase B in untransfected Fao cells (Fao). FaoAB cells (FaoAB) also contained a 41.3 kD aldolase B-immunoreactive protein indicating HAHAB expression. Non-specific background and a 41.3 kD marker for epitope-tagged aldolase B were provided by ts20 and ts20AB cells that lack endogenous aldolase B. HAHAB comigrated with RABM, consistent with 12CA5 and 9E10 epitope constructs being similar in size. Fao cells express over 10 fold more aldolase A than aldolase B (Deschatrette et al., 1979; Deschatrette and Weiss, 1974), so the pattern of labeling was consistent with HAHAB expression equal to or less than total endogenous aldolase (isoforms A + B).

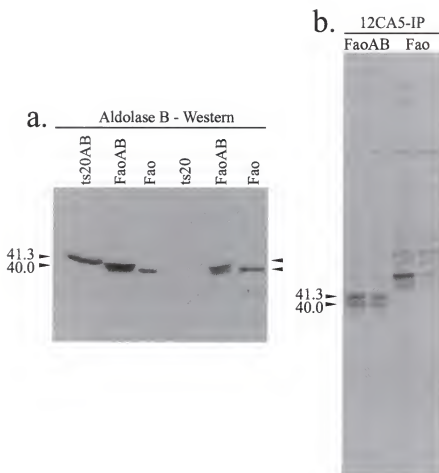


**Figure 4-8: Biochemical Detection of HAHAB.** a) anti-aldolase B western blot analysis was performed on whole cell extracts from the indicated cell lines as described in Materials and Methods;  $\sim 1 \text{ cm}^2$  culture area equivalent loaded per lane from different confluency: ts20AB and ts20 lanes from tightly confluent, first FaoAB and last Fao lanes from recently confluent, remaining lanes from subconfluent; ts20 cells have no endogenous aldolase B, and ts20AB cells express a different epitope-tagged aldolase B, RABM; b) autoradiograph of duplicate anti-12CA5 (HA epitope) immunoprecipitates prepared from  $^{35}\text{S}$ -methionine labeled FaoAB and Fao cultures as described in Materials and Methods. Molecular weights for proteins specifically detected by the methods are indicated.

On autoradiographs of anti-12CA5 immunoprecipitates (12CA5-IP) from cells metabolically labeled with radioactive amino acid, a doublet of HAHAB (41.3 kD) and endogenous aldolase (40.0 kD) was specifically precipitated from FaoAB cells and not Fao cells (4-8b). Since endogenous aldolase lacked 12CA5 immunoreactivity (Fig. 4-7, Fao), the 40 kD aldolase subunits appeared to be associated with HAHAB for immunoprecipitation. This provided evidence that HAHAB subunits tetramerized with endogenous subunits. The results indicated that HAHAB was permanently expressed in a manner expected for a subunit of aldolase. Furthermore, aldolase protein bands migrated in a region of low background on SDS-PAGE of immunoprecipitates facilitating quantification of radioactivity associated with HAHAB (Fig. 4-8b).

In order to compare stress-induced degradation of HAHAB in Fao cells to previous results for RABM in E36 cells (Fig. 4-3 and 4-4), 12CA5-immunoprecipitation was used to isolate and measure the degradation of HAHAB (Fig. 4-9). Starvation increased the degradation of HAHAB by at least two fold (Figs. 4-9 and 4-11) which was even greater than the enhancement of total long-lived protein degradation (Fig. 4-6). Previously, heat stress-induced degradation of RABM (aldolase B) in E36 cells resisted lysosomal inhibitors (Fig. 4-4), but starvation enhanced degradation of HAHAB was completely inhibited by chloroquine (CHQ) or 3-methyladenine (3MA) in Fao cells (Fig. 4-9). The data indicated that HAHAB was degraded in lysosomes via autophagy during starvation, and that cytosolic proteolysis was constant at the constant temperature (37°C) during starvation in E36 cells.

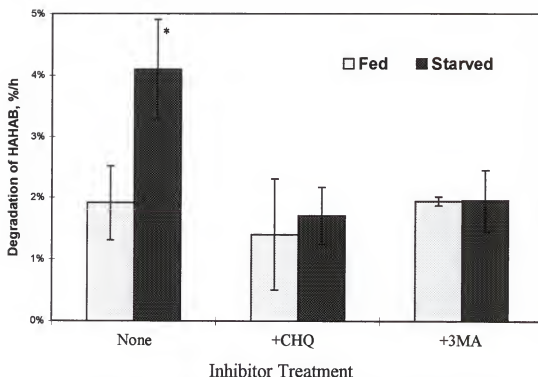


**Figure 4-9: Starvation-Induced Autophagic Degradation of HAHAB in Fao Cells.** FaoAB cells labeled with  $^{35}\text{S}$ -methionine were chased with excess "cold" methionine in DMEM + 10% FBS (Fed) or Krebs-Heinseleit medium without amino acids (Starved) with 160  $\mu\text{M}$  chloroquine (+CHQ), 10 mM 3-methyladenine (+3MA), or no additions (None); radiolabel in immunopurified HAHAB was used to calculate degradation rate (mean  $\pm$  SEM,  $n = 11-23$ ) as described in Materials and Methods; \*, HS > CT, Student's t-test ( $p < 0.0005$ ).

To confirm that HAHAB was representative of endogenous aldolase B, starvation-induced loss of endogenous aldolase B was examined on western blots (Fig. 4-10). When protein synthesis is inhibited protein decreases reflect degradation rates (Henell, et al., 1987). In fed confluent cultures (Fed), aldolase B levels remained constant or slightly increased (statistically insignificant) during 8 hour incubations (Fig.

4-10). Starvation inhibited protein synthesis (data not shown), and endogenous aldolase B levels decreased at a rate that was statistically indistinguishable from the enhanced degradation of HAHAB (Fig. 4-9 and 4-10). 3MA or CHQ inhibited the loss of endogenous aldolase B to a rate of  $\sim 2\%/h$  (Fig. 4-10), consistent with basal cytosolic degradation (Fig. 4-9) and indicating that endogenous aldolase B and HAHAB utilized same starvation-induced autophagic mechanism for degradation.

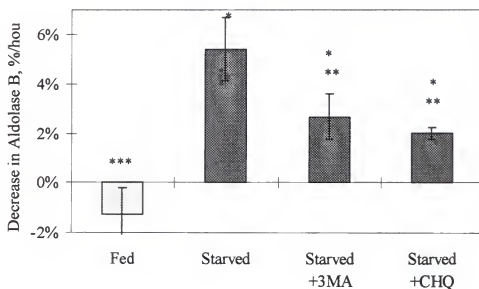


**Figure 4-10: Starvation-Induced Autophagic Degradation of Endogenous Rat Aldolase B in Fao Cells.** Untransfected Fao cells were incubated in the chase media of Figure 4-9 (same labels) for various times, and whole cells directly harvested by boiling in sample loading buffer were analyzed on anti-aldolase B western blots, and changes (mean  $\pm$  SEM,  $n = 16-24$ ) in the level of aldolase B were calculated as previously described (Materials and Methods). Rates of decrease were plotted, so negative value indicates aldolase B increase. \*Starved > Fed (Student's  $t$ -test,  $p < 0.0001$ ); \*\* Starved+Inhibitor < Starved ( $p < 0.0007$ ); \*\*\*Fed  $\cong 0\%/h$  ( $p > 0.15$ ).

### Temperature-Dependent Autophagy and Cytosolic Proteolysis

Due to the temperature-sensitive mutation in ts20 cells, E36 and ts20 cells were routinely maintained at 30.5°C, according to established protocols used here (Gropper, et al., 1991). For the established experimental system, control temperatures were 30.5°C (CT) and heat stress was 41.5°C for one hour then 39.5°C (HS). Heat stress and starvation at 37°C, both, enhanced the complete degradation of total TCA precipitated polypeptides by autophagy (Figs. 3-15 and 4-6). In agreement with this, starvation enhanced the autophagic degradation of immunodetected aldolase B at 37°C (Figs. 4-9 and 4-10). These results indicated that starvation-induced autophagic degradation of aldolase B was rate limiting and faster than cytosolic proteolysis of aldolase B at 37°C in Fao cells. In E36 cells, heat stress increased cytosolic proteolysis of immunoprecipitable aldolase B to a rate greater than heat stress-induced autophagic degradation (Figs. 3-14, 3-15, 4-3, and 4-4). The cytosolic proteolysis, though probably partial, disrupted immunoprecipitation of aldolase B such that degradation of aldolase B appeared to be independent of lysosomal degradation.

To determine whether heat stress-induced cytosolic proteolysis of aldolase B was a general phenomenon beyond E36 cells, the heat stress and starvation protocols were compared using Fao cells (Fig. 4-11). Consistent with E36 cells, 39.5°C heat (stress) caused increased proteolysis of HAHAB relative to 30.5°C control temperature (unstressed) in Fao cells (Fig. 4-11, Heat Stress Induction, untreated). The proteolysis

enhanced by 39.5°C was resistant to chloroquine treatment (CHQ), indicating the presence of the enhanced cytosolic mechanism (Fig. 4-11, Heat Stress Induction). Sister

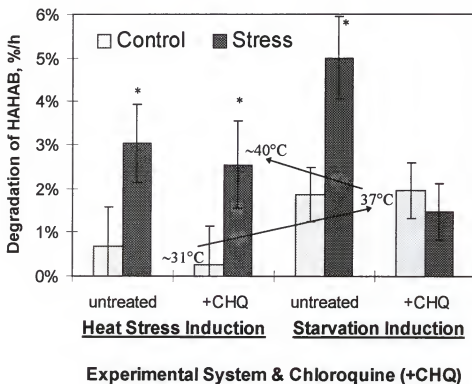


**Figure 4-11: Temperature Increases Cytosolic Proteolysis and Starvation Induces Lysosomal Proteolysis of Aldolase B (HAHAB).** Heat stress and starvation induction of protein degradation in Fao cells and degradation of HAHAB was done as previously described above (mean  $\pm$  SEM,  $n = 14-16$ ). "Control" and "Stress" treatments are different for the two induction systems. The degradation rates are estimates of  $k_d$  for HAHAB proteolysis. Student's t-test: \*, Stress > Control ( $p < 0.001$ ). Arrows connect temperatures that indicate  $k_d$  estimates for "cytosolic" (CHQ-resistant) proteolysis.

cultures of the Fao cells confirmed that starvation-induced autophagic degradation of aldolase B occurred at 37°C when amino acids and additional nutrients were withheld (Fig. 4-11, Starvation Induction). For the starvation-induced degradation at 37°C, it

was confirmed that inducible lysosomal degradation was inhibited by chloroquine treatment (Fig. 4-11, Starvation Induction,  $\pm$ CHQ). Altogether, the data demonstrated that cytosolic proteolysis of aldolase B can occur in different cell types (E36 Chinese hamster lung and Fao rat hepatoma) with different aldolase B constructs (RABM and HAHAB), and is temperature-dependent.

The degradation measurements for aldolase B represent rate constants ( $k_d$ ) set by rate-limiting mechanisms. For cytosolic proteolysis of HAHAB, the rate limiting step increased proportionally with increased temperature (Fig. 4-11, arrows and temperatures). This suggested that increased reactivity was by direct thermal stimulation of a rate-limiting cytosolic mechanism. To test this possibility, the  $k_d$ 's that were determined for three different temperatures (30.5°C, 37°C, and 39.5°C) were used to make Arrhenius plots (Fig. 4-12) to test this possibility.

Evidence derived from the Arrhenius plots (Fig. 4-12) is limited by the fact that only three temperatures were available. Total cellular degradation of long-lived proteins in cultured smooth muscle cells was previously shown to undergo direct thermal stimulation with  $E_a = 18$  kcal/mole at 15-37°C (Bates, et al., 1982). With similar data here (Total TCA), an  $E_a = 19.6 \pm 1.5$  kcal/mole at 30.5-39.5°C was calculated and was statistically indistinguishable ( $p > 0.1$ ) from the previously published result (Fig. 4-12). This argued that our calculations gave reasonable estimates of  $E_a$ .

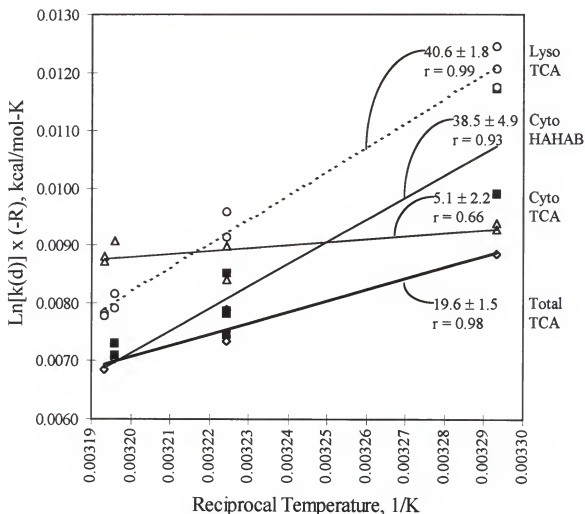


**Figure 4-12: Heat Stress Induces Cytosolic Proteolysis of Aldolase B By Thermal Stimulation of Lysosomes.** Cytosolic (Cyto, light unbroken line) or lysosomal (Lyso, broken line) proteolysis was degradation resistant or sensitive, respectively, to inhibition with 3-methyladenine or chloroquine; the sum of these (Cyto+Lyso) represents total cellular proteolysis (Total, heavy unbroken line); degradation rates for immunoprecipitated aldolase B (HAHAB, filled symbols) or TCA precipitated polypeptides (TCA, open symbols) were used as  $k_a$ -estimates to construct Arrhenius plots with slope = activation energy in kcal/mole ( $E_a$ ), an arc connects estimated slope,  $E_a$  (mean  $\pm$  SEM,  $n = 7$  or 8) accompanied by the corresponding correlation coefficient ( $r$ ) for a least squares linear plot; square represents cytosolic proteolysis of HAHAB; triangle, circle, or diamond represents cytosolic, lysosomal, or total proteolysis of long-lived proteins, respectively. By Student's  $t$ -test, all slopes were much  $> 0$  and different from each other ( $p < 0.0004$ ), except "Cyto TCA" was close but slightly  $> 0$  ( $p < 0.028$ ), and "Cyto HAHAB" slope was indistinguishable from "Lyso TCA" ( $p > 0.53$ ).

Based on sensitivity to inhibitors, lysosomal (Lyso) and cytosolic (Cyto) degradative mechanisms for long-lived proteins were separated (Fig. 4-12, TCA). Both had linear Arrhenius plots, giving  $E_a = 40.6 \pm 1.8$  and  $5.1 \pm 2.2$  kcal/mole for lysosomal and cytosolic degradation, respectively. A plot for the cytosolic proteolysis of HAHAB (aldolase B) was linear confirming that heat stress induced degradation could be accounted for by thermodynamic effects of temperature. The cytosolic proteolysis of HAHAB had an  $E_a = 38.5 \pm 4.9$  kcal/mole which was much larger than for cytosolic proteolysis of TCA precipitable polypeptides but closely corresponded to the  $E_a$  of a lysosomal mechanism reported above, as well as the lysosomal degradation of endocytosed apolipoprotein B,  $E_a = 35$  kcal/mole at 20-37°C (Bates, et al., 1982). Together results suggested that "cytosolic" (3MA or chloroquine resistant) proteolysis of aldolase B (HAHAB) was independent of autophagic sequestration (inhibition) and acidic lysosomal pH but occurred by a complex mechanism with  $E_a$  similar to lysosomal degradative mechanisms.

Lysosomal degradation of long-lived proteins is generally thought to be limited by sequestration rate (high  $E_a$ ), and sequestration can occur by non-autophagic (possibly 3MA-resistant) process of receptor recognition and translocation (Dice and Chiang, 1988; Cuervo, et al., 1996). Chloroquine neutralizes the acidity of lysosomes and inhibits lysosomal acid hydrolases, but proteolysis of HAHAB continued in the presence of this drug. Consistent with this, aldolase A and B are thought to undergo limited proteolysis by a neutral lysosomal peptidase (Horecker, et al. 1985). Though the data

are not conclusive, past investigators have shown that this peptidase might occur on the cytosolic surface of lysosomes (Pontremoli, et al., 1982) which in itself could confer resistance of the peptidase activity to 3MA and CHQ. Given these possibilities, 3MA or CHQ resistant "cytosolic" proteolysis for aldolase B (HAHAB) in Figure 4-12 might actually occur by lysosomal mechanisms. The highest  $E_a$  found published for cytosolic proteolysis was  $E_a = 27 \pm 5$  kcal/mole reported for an enzyme now established as the 20S proteasome (Hough et al., 1986; Bond and Butler, 1987; Waxman, et al., 1987; Coux, et al., 1996).  $E_a$  for HAHAB proteolysis was significantly greater than this ( $p < 0.019$ ), suggesting a more complex mechanism like sequestration into lysosomes. The mechanism of 3MA and chloroquine resistant proteolysis of aldolase B (HAHAB) remains unidentified, but the results support that heat stress increases both lysosomal and cytosolic mechanisms for the degradation of aldolase B by direct thermodynamic stimulation of elevated temperatures.

#### A Model For the Degradation of Aldolase B

Figure 4-13 summarizes the results of the analysis above in a hypothetical model for the degradation of aldolase B. It previously was established (Chapter 1) that starvation for amino acids and serum (S) can induce autophagic degradation of aldolase A and B (Fig. 4-13, B1→C→D). In Chapter 3, evidence was provided that starvation and heat stress (HS) induced ubiquitin-mediated autophagy (Fig. 4-13, A→B2→C1→C2→D). Since it appeared that ubiquitinated aldolase B was enriched in isolated autophagic vacuoles and lysosomes relative to cytosol, sequestration of aldolase

B appears to be enhanced by ubiquitination ( $B2 > B1$ ). Ubiquitination is known to mediate cytosolic proteolysis of proteins into 6-9 amino acids long peptides (Coux, et al., 1996; Lowe, et al., 1995) indicated by  $A \rightarrow E$  (lower arrow?)  $\rightarrow F$  (Fig. 4-13). Though aldolase B occurs in ubiquitinated forms, when ubiquitination was inhibited (heat stressed ts20 cells) an enhance cytosolic (3MA and CHQ resistant) proteolysis of aldolase B still occurred (Fig. 4-13,  $A \rightarrow E$  (upper arrow)  $\rightarrow F$ ).

The proteolysis at step F increased with temperature consistent with direct thermal enhancement of chemical reactivity. The estimated activation energy ( $E_a \cong 35$ -40 kcal/mole) was closer to a known value for a lysosomal pathway ( $E_a = 35$  kcal/mole) than for the major cytosolic protease, the 20S proteasome ( $E_a = 27$  kcal/mole). However, there was no difference from the 20S proteasome at a 0.01 significance level. Furthermore, a basal level of ubiquitination can be measured in ts20 cells (Gropper, et al., 1991), and ubiquitination is thought to be rate limiting ( $E_a > 27$  kcal/mole) for proteosomal degradation (Coux, et al., 1996), indicating that step F proteolysis might still occur in the proteasome. Previously proposed mechanisms for limited proteolysis of aldolase B on the cytosolic surface of lysosomes (Pontremoli, et al., 1982) and for non-autophagic sequestration into lysosomes (Dice and Chiang, 1988) argue that the high  $E_a$  of step F proteolysis might still involve lysosomes. Current data is insufficient to definitively distinguish these possibilities. Here, cytosolic proteolysis was preferred as the most likely possibility.

At higher temperatures, cytosolic proteolysis of aldolase B (Fig. 4-13, step F) becomes equal to or greater than sequestration into lysosomes (step B). This

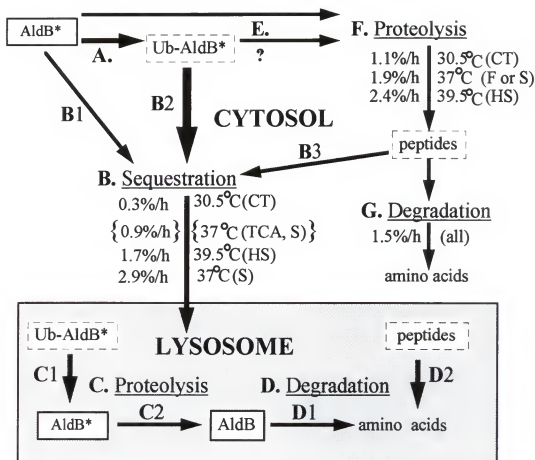


**Figure 4-13: Pathways for Degradation of Aldolase B.** Large stippled box, lysosomal compartments (autophagic vacuoles and lysosomes); small box, aldolase B polypeptide maintaining predominately native (solid outline) or denatured (broken outline) conformation; AldB\*, full length active enzyme; AldB, proteolytically processed inactive enzyme; Ub-AldB, ubiquitinated aldolase B; peptides, hypothetical fragments of aldolase B; arrows and upper case bold face letters, processes discussed in text; %/h, median fractional rate of flux (SEM  $\leq 1/6$  of magnitude shown,  $n = 24-43$ ) through the indicated process at temperature and condition: CT, control temperature; HS, heat stress; F, fed; S, starved; "Proteolysis" indicates cleavage in to polypeptides; "Degradation" indicates complete proteolysis to amino acids (TCA soluble). {37°C (TCA, S)}, rate of autophagic sequestration at 37°C for total TCA precipitable polypeptides during starvation.

interpretation was made because during heat stress aldolase B degradation was independent of autophagic degradation in lysosomes. Since TCA precipitable polypeptides were still reliant on autophagic degradation, step F was considered to produce peptides, consistent with involvement of the proteasome. Complete cytosolic proteolysis to amino acids occurred (Fig. 4-13, step G) which was fairly constant with a low temperature dependency,  $E_a = 5.1$  kcal /mole, that was temperature-independent at a 0.01 significance level (Fig. 4-12, Cyto TCA). The balance of peptides produced in step F are sequestered (step B3) and degraded (step D2) in lysosomes. According to TCA-precipitable polypeptides, the autophagic degradation rate during starvation (TCA, S) was 0.9%/h consistent with the temperature-dependency shown in Figure 4-12 (Lyso TCA). However, in the same cells (Fao) under the same treatment (starvation), lysosomal degradation of aldolase B was threefold higher, 2.9%/h (Fig. 4-13, step B, (S)). This indicates that lysosomal mechanisms have a preference for degrading aldolase B over TCA precipitated total proteins, suggesting an additional mechanism. In Chapter 5, a role for receptor-mediated targeting to lysosomes is demonstrated for aldolase B.

## CHAPTER 5: SIGNAL-MEDIATED DEGRADATION OF ALDOLASE B

### Introduction

Chapter 1 introduced the hypothesis that stress-induced degradation of aldolase B requires ubiquitination and receptor-mediated targeting (Fig. 1-4). In Chapter 3, evidence was presented that aldolase B was ubiquitinated and enriched in lysosomes for a ubiquitin-mediated autophagic degradation during starvation and heat stress. In Chapter 4, temperature increase caused an increase in cytosolic and lysosomal degradation consistent with direct thermal stimulation of chemical reactivities. During starvation for amino acids and serum, autophagic degradation of total TCA precipitated polypeptides was consistent with the temperature-dependent rate predicted by thermal stimulation at 37°C (Fig. 4-13, step B).

Lysosomal degradation of aldolase B in Fao cells was threefold higher than for TCA precipitated polypeptides (Fig. 5-1), suggesting a mechanism preferential for aldolase B sequestration into lysosomes. In Figure 5-1, the total height of each bar indicates the total degradation rate during starvation for the indicated proteins, including degradation rates for total long-lived proteins (first bar, Total), permanently expressed recombinant aldolase B (third bar, HAHAB), and rate of loss for endogenous aldolase B estimated on western blots (second bar, aldolase B). Each bar is divided into two parts.

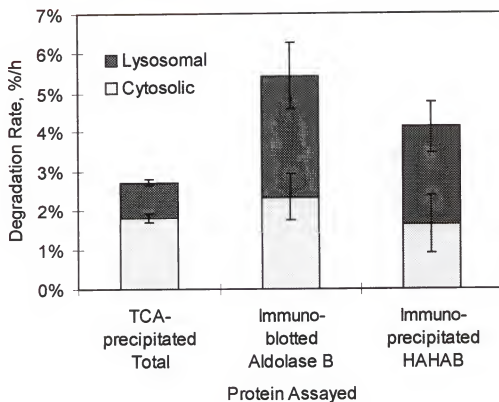


**Figure 5-1: Starvation-Induced Lysosomal Degradation is faster for Aldolase B than for Other Long-lived Proteins.** Starvation-induced degradation was measured at 37°C as previously described (Figs. 4-6, 4-9, and 4-10). Degradation of protein which was resistant to lysosomal inhibition is superimposed on the total starvation-induced rate to show the relative contribution of degradation (mean  $\pm$  SEM,  $n = 8-24$ ) occurring in cytosol (cytosolic) and lysosomes (lysosomal). All cytosolic degradation rates were comparable to each other; lysosomal degradation for Aldolase B or HAHAB was greater than TCA-precipitated (Student's  $t$ -test,  $p < 0.001$ ).

The lower part indicates the degradation rate contributed by proteolysis in the cytosol (Fig. 5-1, cytosolic, light-shaded part). These rates were calculated by inhibiting lysosomes during starvation. They agreed with each other and were not statistically different from basal degradation under fed conditions (Fig. 4-9). The results suggest that cytosolic degradation of aldolase B probably follows a common basal mechanism used

by most long-lived proteins, consistent with the major role proposed for the cytosolic proteasome (Coux, et al., 1996). Further, this indicated that in the presence of amino acids (fed conditions) basal lysosomal degradation was too low to detect in Fao hepatoma cells, consistent with the ability of liver-derived cells to respond to amino acid concentrations by down-regulating protein degradation (Hendil, et al., 1990).

In Figure 5-1, the upper part of each bar represents starvation-induced degradation that occurred in lysosomes (lysosomal, dark-shaded part) calculated by the degradative rate lost by treatment with lysosomal inhibitors. Lysosomal degradation accounted for the increase in protein degradation caused by starvation. During starvation, lysosomal degradation of total long-lived proteins (TCA-precipitated) was about 0.9%/hour, but lysosomal degradation of immunodetected aldolase B was much higher at 2.9%/h. This indicated that aldolase B utilized an additional lysosomal mechanism (relative to most long-lived proteins) that mediated ~2%/h greater rate of proteolysis.

Intralysosomal degradation is rapid and limited by the rates of delivery of proteins into lysosomes. Starvation can enhance two mechanisms for delivery of cytosolic proteins into lysosomes: bulk uptake by autophagy and selective uptake by a receptor-mediated mechanism. Above and in previous chapters, autophagic mechanisms were considered. In this chapter, evidence demonstrates the existence of a lysosomal targeting signal in aldolase B that specifically mediates starvation-induced degradation. Lysosomal targeting signals have been characterized for other cytosolic proteins that

follow receptor-mediated delivery to lysosomes. A description of the proposed mechanism for receptor-mediated targeting is presented in Chapter 1. Aldolase B had characteristics of substrate proteins for this degradative mechanism, including three potential targeting signal motifs (Fig. 1-2). These targeting motifs were mutated, and starvation induced degradation of mutant protein was compared to wildtype. Below, glutamine #111 is shown to be essential for the starvation-induced degradation of aldolase B.

#### Transient Expression of RABM Mutations in Putative Lysosome Targeting Signals

In order to examine a role for three potential lysosomal targeting signals in the aldolase B sequence (Fig. 1-2), plasmid vectors for expressing RABM (rat aldolase B with a carboxyl terminal myc epitope tag) were altered by site-directed mutagenesis to change an "essential" glutamine (Q) in each signal to either threonine (T) or asparagine (N) (Table 5-1). The three "essential" glutamines occurred at defined amino acid positions in the aldolase B sequence: residue numbers 12, 58, and 111 (start methionine = 1). A three letter "mutation code" was adopted here to label the data such that the amino acid at positions 12, 58, and 111 were represented in order by the single letter amino acid convention. In this manner, QQQ indicated wildtype, and QQN indicated that Q111 was changed to asparagine (N).

At least seven site-directed mutant RABM's representing all mutant permutations needed to be expressed in cells at sufficient levels for biochemical detection. Given the same efficiency as for the isolation of the FaoAB cell line (one permanent expressing line per 340 G418-resistant Fao clones), 2,000-3,000 transfected lines would need isolated,

**Table 5-1: Site-Directed Mutations in Aldolase B Sequence for Potential Signal Motifs of Receptor-Mediated Targeting to Lysosomes**

| Labels Used           |               | Amino Acid Residue** at the Essential Glutamine Position for Each of Three Potential Motifs |            |             |
|-----------------------|---------------|---------------------------------------------------------------------------------------------|------------|-------------|
| Protein Studied*      | Mutation Code | Site 1 #12                                                                                  | Site 2 #58 | Site 3 #111 |
| HAHAB                 | QQQ (wt)      | Q                                                                                           | Q          | Q           |
| RAB or Fao endogenous | QQQ (wt)      | Q                                                                                           | Q          | Q           |
| RABM or RABMQQQ       | QQQ (wt)      | Q                                                                                           | Q          | Q           |
| RABMTQQ               | TQQ           | T                                                                                           | Q          | Q           |
| RABMQTQ               | QTQ           | Q                                                                                           | T          | Q           |
| RABMQNQ               | QNT           | Q                                                                                           | N          | Q           |
| RABMQQT               | QQT           | Q                                                                                           | Q          | T           |
| RABMQQN               | QQN           | Q                                                                                           | Q          | N           |
| RABMTTQ               | TTQ           | T                                                                                           | T          | Q           |
| RABMTQT               | TQT           | T                                                                                           | Q          | T           |
| RABMQTT               | QTT           | Q                                                                                           | T          | T           |
| RABMTTT               | TTT           | T                                                                                           | T          | T           |

\*RAB, rat aldolase B; HAHAB, human aldolase B (97% identical to RAB; Fig. 1-2); HAHAB,

HAB with 12CA5 epitope at amino terminus; RABM, RAB with 9E10 epitope at C-terminus

\*\*Single letter convention

amplified, and screened for permanent expression. To avoid this, other candidate cell lines were screened for transfection efficiency (Table 5-2). Transfection efficiency was defined as the fraction of cells expressing RABM by immunofluorescence screening. Some commercial transfection lipid formulations were also screened and found that

transfection lipids operated differentially between cell lines. For example, Pfx-5 appeared to effectively transfect BHK cells but in other cell lines was toxic, produced an inclusion body artifact, or failed to express RABM (Table 5-2).

The HuH7 human hepatoma cell line transfected with Pfx-3 (Invitrogen) had the highest relative transfection efficiency for expressing RABM (Table 5-2), so isolation of HuH7 cell lines permanently expressing RABM was attempted. Hundreds of HuH7 colonies isolated after transient transfection acquired resistance to the selectable marker (G418), but none of forty clonal cell lines retained sufficient expression of recombinant aldolase B for biochemical studies. This was consistent with the low incidence of permanent expression of recombinant aldolase B in Fao cells, indicating a need for massive screenings. However, the transient

**Table 5-2: Optimization of Cell Line and Lipid Type for Transient Expression of RABM\***

| LIPID | Cell Lines |            |            |          |
|-------|------------|------------|------------|----------|
|       | Fao        | HuH7       | NRK        | BHK      |
| Pfx-1 | toxic      | toxic      | 0%         | 0.4-0.5% |
| Pfx-2 | toxic      | toxic      | 0%         | 1-2%     |
| Pfx-3 | toxic      | 2-3%       | <0.05%     | 0%       |
| Pfx-4 | toxic      | toxic      | 0%         | 0.3-0.4% |
| Pfx-5 | toxic      | inclusions | 0%         | 0.2-0.3% |
| Pfx-6 | toxic      | toxic      | <0.05%     | 1-2%     |
| Pfx-7 | 0% ?       | n/d        | <0.05%     | 0.5-1%   |
| Pfx-8 | inclusions | inclusions | inclusions | 0.5-1%   |
| DOTAP | toxic      | 1-2%       | <0.05%     | 0.3-0.4% |

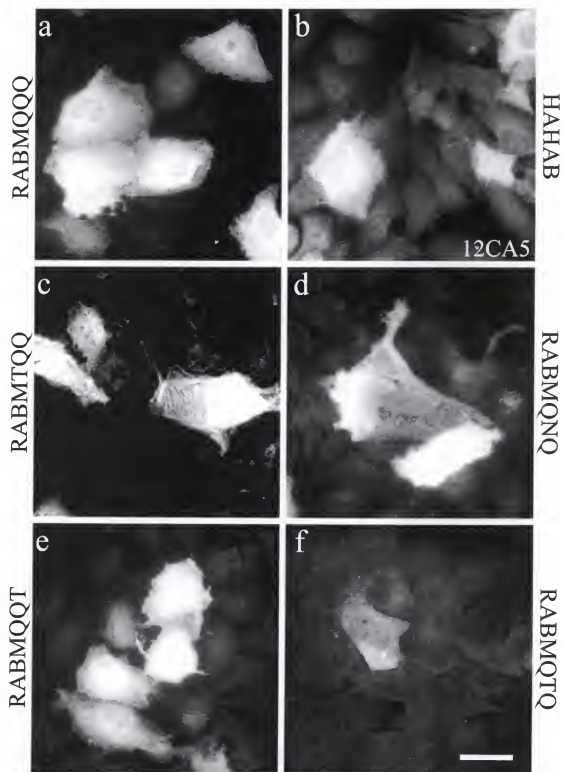
\*, fraction of cells immunofluorescently labeled for RABM-specific expression; toxic, >50% cells had wrinkled appearance, obvious sloughing off present; inclusions, large fluorescent inclusion bodies with little or no cytosolic labeling.

expression of RABM in HuH7 cells was relatively high and after optimization appeared sufficient for biochemical assays (Table 5-3). Commonly, protocols call for plating cells the day before transfection, but isolated individual HuH7 cells in cultures one day after plating were killed by Pfx-3 treatment (data not shown) which limited transfection efficiency (Table 5-3). By incubating cells for two days after plating, most cells contacted other cells, survived Pfx-3 mediated transfection, and transfection efficiency was increased by tenfold (Table 5-3). Though not generally examined during optimization of transfection, the incubation time between plating cells and transfection was found to have a large effect on transfection efficiency. Consistent transient transfection efficiency of >15% (Table 5-3) indicated that transient transfection could replace the more laborious isolation of permanent cell lines.

| <b>Table 5-3: Effects of Timing and Lipid:DNA Ratio on Transfection Efficiency for RABM Expression in HuH7 Cell Line</b> |                          |                                                      |            |            |
|--------------------------------------------------------------------------------------------------------------------------|--------------------------|------------------------------------------------------|------------|------------|
| <b>Time After Plating</b>                                                                                                | <b>Transfection Time</b> | <b>Pfx-3 Lipid:DNA Ratio<br/>(DNA held constant)</b> |            |            |
| <b>days</b>                                                                                                              | <b>hours</b>             | <b>3:1</b>                                           | <b>6:1</b> | <b>9:1</b> |
| <b>1</b>                                                                                                                 | <b>4</b>                 | n/d                                                  | 2%         | n/d        |
| <b>2</b>                                                                                                                 | <b>4</b>                 | 20%                                                  | 21%        | 21%        |
| <b>2</b>                                                                                                                 | <b>8</b>                 | 27%                                                  | 22%        | 23%        |

In order to compare degradation rates for wildtype and mutant RABM, expression levels for the mutants had to be sufficient. HuH7 cell transfection efficiencies

Figure 5-2: Transient Expression of Recombinant Aldolase B in HuH7 Cells. HuH7 human hepatoma cells were transfected for optimum transient expression of various epitope-tagged aldolase B proteins defined in Table 4-1, processed for epitope-tag specific immunofluorescence microscopy (9E10 for all, except 12CA5 for panel b), and micrographs exposed until background labeling just became apparent; panels a through e, representative fields showing labeling patterns for recombinant aldolase B proteins defined in Table 5-1, similar fields occurred with expression of all the different proteins except RABM's with threonine (T) at position #58; panel f, expression of QTQ mutant RABM showing the brightest cell on an entire coverslip, only rare cells were clearly brighter than background but dim out-lining of nuclei was visible in many cells that did not occur on untransfected control coverslips (not shown). Scale bar = 50  $\mu$ M.



were consistently greater than 15% for RABM proteins (Fig. 5-2, panels a-f) using wildtype (QQQ) and site-directed mutants with changes in amino acid residues #12 or #111 (TQQ, QQT, QQN, and TQT). Such high levels of expression were sufficient for isolation by immunoprecipitation which confirmed comparable expression levels between wildtype RABM and these mutants (Fig. 5-3). Note, the transiently expressed proteins were similar for different mutants and wildtype RABM (41.3 kD) and did not occur in cells transiently expressing  $\beta$ -galactosidase ( $\beta$ Gal). Previously during permanent expression, RABM became associated with endogenous 40 kD aldolase which could be co-immunoprecipitated (Fig. 4-8b). However, immunoprecipitation from transiently transfected cultures pelleted very little or no 40 kD protein, indicating that transient protein expression per cell was so high relative to endogenous aldolase expression that the exogenous RABM predominately associated with itself presumably forming homo-oligomeric complexes of RABM. Since transient overexpression excluded association with endogenous aldolase, possible complications from oligomeric interactions with endogenous aldolase subunits were reduced. For example, if all subunits of an aldolase tetramer are degraded together, then wild type signals in an endogenous subunit could rescue an associated mutant subunit. Transient expression seems to eliminate this possibility. The results indicated that wildtype RABM and mutants at residues #12 and #111 were expressed sufficiently for protein degradation measurements.

Replacing glutamine #58 with threonine (QTQ, TTQ, QTT, or TTT) prevented high RABM expression (Fig. 5-2, panel f), and further biochemical analysis could not be

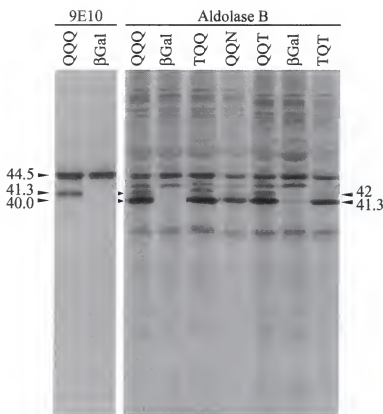


**Figure 5-3: Immunoprecipitation of RABM and its Mutants Transiently Expressed in HuH7 Cells.** HuH7 cell cultures were transfected for transient expression of  $\beta$ -galactosidase ( $\beta$ Gal) or RABM forms identified by the mutation codes shown in Table 5-1; cells were then metabolically labeled with  $^{35}\text{S}$ -methionine then processed for anti-myc epitope (left panel, 9E10) or for anti native aldolase B (right panel, Aldolase B) immunoprecipitation as described in Materials and Methods; autoradiographs of dried gels of immunoprecipitation pellets run on SDS-PAGE are shown; left and right panels are lined up on a 44.5 kD heavy background protein (actin?), and differences in electrophoretic migration between gels are indicated by relative positions of the 41.3 kD band relative to the 44.5 kD band.

done (summarized later in Table 5-4). The minimal change in the cDNA (2 base pairs) is unlikely to affect transfection efficiency. Consistent with this, many very low expressing cells were apparent by very dim but visible negative nuclei suggesting that transfection occurred, but the protein was not expressed well. To get around this low expression, a less harsh change of glutamine #58 to asparagine (N) was tried which successfully produced enough protein for degradation assays (Figs. 5-2, panel d, QNQ). In the QNQ mutant, an amide side chain chemistry was retained at residue #58, suggesting a possible role in aldolase B protein stability.

Other evidence supports a role for glutamine #58 in the structural stability of aldolase B. First, glutamine #58 is embedded in the tertiary structure of the protein, placing it in the right place for such a role. Glutamine #58 is not a highly conserved residue which argues against this role. However, neighboring amino acid sequence was examined in different mammalian aldolase isoforms and found that when glutamine #58 was missing then glutamine occurred at a position three residues away. These glutamine side chains would occur on the same side of an  $\alpha$ -helix in a similar 3-D position and could perform the same structural role. Supporting this idea, glutamine #58 occurs in an  $\alpha$ -helical region of aldolase structure.

The results here provided wildtype and mutant RABM proteins transiently expressed in HuH7 cells at similar levels (Figs. 5-2 and 5-3), allowing effects on starvation induced degradation to be examined for mutations at all three potential signal sequence motifs found in aldolase B.

### Starvation Induces Autophagic Degradation in HuH7 Cells

Before examining transiently expressed aldolase B (RABM), starvation-induced degradation of total long-lived proteins in HuH7 cells was examined. Chloroquine (CHQ) but not 3-methyladenine (3MA) significantly reduced basal degradation of long-lived proteins in HuH7 cells (Fig. 5-4). This suggested that a fraction of unstressed basal protein degradation occurred in lysosomes but not by 3MA-sensitive autophagy. Both starvation-induced

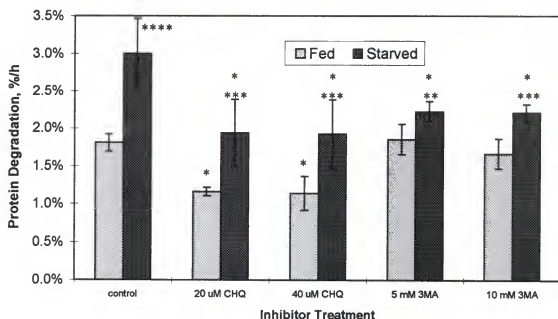


**Figure 5-4: Starvation-Induced Autophagic Degradation of Long-lived Proteins Occurs in HuH7 Cells.** HuH7 cultures were metabolically labeled with  $^{14}\text{C}$ -valine, chased with 10 mM “cold” valine in DMEM+10% FBS (Fed) or Krebs-Heinseleit without amino acids (starved), and loss of TCA precipitable counts was used to calculate degradation rates (mean  $\pm$  SEM,  $n = 12$ ) as described in Materials and Methods; media were unsupplemented (control) or supplemented with indicated concentrations of chloroquine (CHQ) or 3-methyladenine (3MA); Student’s t-tests: inhibitor treated < corresponding control degradation rate (\*,  $p < 0.013$ ); Starved > Fed degradation rate (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ , \*\*\*\*,  $p < 0.0001$ ).

(Starved) and basal (Fed) degradation of long-lived proteins was inhibited with chloroquine (CHQ) such that starvation appeared to induce a mechanism that was resistant to CHQ (Fig. 5-4). Alternatively, the results were consistent with mechanisms for fed and starved degradation having a similar sensitivity to CHQ. Generally, lysosomal degradation plays a greater role in starvation-induced degradation (Starved) than in basal degradation (fed). Since the results were inconsistent with this, HuH7 cells might have a non-specific sensitivity to CHQ which affects both basal and starvation-induced degradation equally. Doubling inhibitor concentration did not change results (Fig. 5-4), indicating that a maximal effect occurred, but the possibility that lower CHQ concentrations might be more specific for starvation-induced degradation in HuH7 cells was not examined. 3-methyladenine specifically reduced the enhanced degradation of total long-lived proteins by about 0.8%/h in HuH7 cells (Fig. 5-2), consistent with the 0.9%/h inhibition of enhanced autophagic degradation in Fao cells (Fig. 4-6). The results suggested that starvation-induced autophagic degradation of total long-lived proteins occurred in HuH7 cells.

#### Transient Expression Does Not Affect Starvation-Induced Degradation of RABM

I showed that HuH7 cells undergo starvation-induced degradation of long-lived proteins and that 9E10 myc-epitope tagged rat aldolase B (RABM) could be transiently expressed at sufficient levels for biochemical analysis. In this section, the degradation of the transiently expressed RABM is shown to be comparable to permanently expressed recombinant aldolase B (HAHAB) and endogenous aldolase B of Fao cells.

I found that variation in permanent expression levels had little or no effect on degradation rates in E36 cells (data not shown), but a sub-population of the transiently transfected HuH7 cells clearly had higher RABM levels per cell than previously examined. Thus, it was important to establish whether transient transfection altered the degradation of

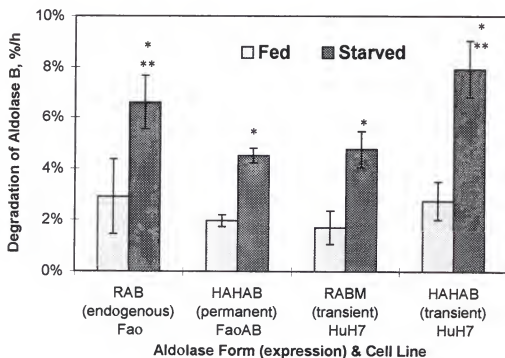


**Figure 5-5: Differences in Expression and Epitope Tag Have No Effect on Starvation-Induced Degradation of Aldolase B in Hepatoma Cell Lines:** Cultures of cell lines indicated expressing aldolase B were measured for fed and starved aldolase B degradation rates  $\pm$  standard error ( $n = 25$  to  $37$ ) according to protocols compatible for each form of the enzyme as described in Materials and Methods. RAB (endogenous) Fao, endogenous rat aldolase B in Fao rat hepatoma cells; HAHAB (permanent) Fao, permanently expressed amino HA-tagged human aldolase B transfected in Fao cells; RABM (transient) HuH7, transiently expressed carboxyl Myc-tagged rat aldolase B in HuH7 cells; HAHAB (transient) HuH7, transiently expressed HAHAB in HuH7 human hepatoma cells. Student's t-test: Starved > Fed (\*,  $p < 0.0005$ ); HAHAB (Starved) in HuH7 > in FaoAB and RAB (starved) in Fao > RABM in HuH7 (\*\*,  $p < 0.001$ )

aldolase B. Figure 5-5 compares the basal and starvation-induced degradation of various forms of aldolase B expressed in various ways. The results are similar regardless of the variations. There was little or no effect caused by epitope tagging. Tagging at the amino terminus or the carboxyl terminus gave identical results, and using 12CA5 versus 9E10 epitope also had no effect. Whether endogenously expressed, permanently expressed from transfected cDNA, or transiently expressed as described above the results were similar (Fig. 5-5).

#### Site-Directed Mutations Did Not Affect Wildtype Activity of RABM

Endogenous 40 kD aldolase was absent in proteins immunoprecipitated from transiently transfected cells (Fig 5-3), indicating that characteristics of immunoprecipitated RABM could be examined in isolation from endogenous aldolase effects. Antiserum against aldolase B also failed to pull down endogenous aldolase indicating that the endogenous isoform of HuH7 cells is probably aldolase A (Fig. 5-3). These results indicated that exogenous RABM is for the most part separate from endogenous aldolase, during transient expression.

Even though endogenous aldolase was not associated with transiently overexpressed RABM, a 42 kD protein was specifically immunoprecipitated from RABM expressing but not  $\beta$ -galactosidase expressing cells (Fig. 5-3). This indicated that RABM associated with an endogenous 42 kD protein. Aldolase isozymes have an established binding affinity for

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which has a molecular weight of 42 kD. The results suggest that RABM retains GAPDH binding activity. If so, then all the mutants examined had no effect on GAPDH binding, because the 42 kD protein was co-immunoprecipitated with them as well (Fig 5-3).

With transient expression, immunoprecipitation was used to isolate epitope-tagged aldolase B away from endogenous aldolase, giving purified RABM or HAHAB. By measuring activity specifically precipitated by the epitope tag, the effect of site-directed mutations on the enzymatic activity of aldolase B could determine. As shown in Figure 5-6, all forms of recombinant aldolase B tested had similar activity regardless of epitope tag or site-directed mutations. Thus, effects of mutations on starvation-induced degradation shown below are not due to indirect effects of altered aldolase B activity (Fig. 5-6).

#### Glutamine Residue #111 is Required for Starvation-Induced Degradation of Aldolase B

Above, the degradation of RABM transiently expressed in HuH7 cells was shown comparable to the degradation of permanently expressed HAHAB and endogenous RAB (Fig. 5-5). These aldolase B forms followed a starvation inducible pathway for degradation. Then mutant forms of RABM were transiently expressed in HuH7 cells; the different mutations are described and defined in Table 5-1.

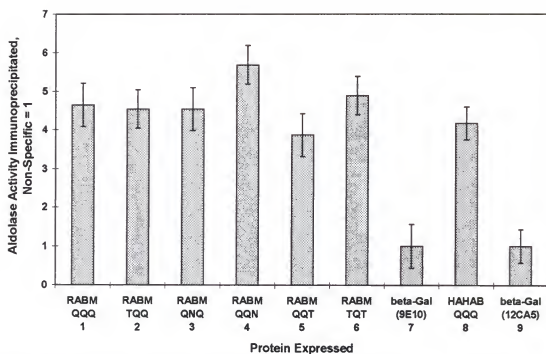


**Figure 5-6. Different Forms of Recombinant Aldolase B Retain Similar Levels of Catalytic Activity.** Replicate HuH7 cell cultures were transfected for transient expression of indicated proteins, lysed in 1% NP40/1 mM EDTA/TBS pH 7.5, and immunoprecipitated for 9E10 (bars 1 through 7) or 12CA5 (bars 8 and 9) epitopes as detailed in Materials and Methods under subsaturating conditions. After standard washes, pellets were washed with TE pH 7.5 then suspended in 1 ml aldolase reaction mixture. Periodically, each sample was pelleted and OD(340 nm) of supernatant was measured. The mean  $\pm$  SEM ( $n = 3$ ) OD decrease caused by adding FDP was normalized to the non-specific background of cultures expressing  $\beta$ -galactosidase and immunoprecipitated with the same antibody (bars 7 or 9). Student's t-test: relative activity for RABM or HAHAB expressing cultures > untransfected non-specific controls, lanes 7 and 9 ( $p < 0.0001$ ).

Figure 5-7 shows that glutamine #111 is essential for starvation-induced degradation of RABM. Changing at glutamine #12 to threonine (TQQ) yielded fed and starved degradation rates for RABM that were statistically indistinguishable from wildtype aldolase B sequence (QQQ). Even though the peptide sequence at glutamine #12 is the most conserved of the mutated signals (identical in all vertebrate aldolases), it

did not function as a signal for starvation-induced degradation (Fig. 5-7). A role for glutamine #58 could not be assessed by threonine substitution, because this resulted in expression too low for degradation assays. When glutamine was replaced with asparagine at residue #58 (QNQ), conserving an amide at this residue, expression was close to other high level transient expressions, and starvation-induced degradation was like wildtype (Fig. 5-7). The results indicated that glutamines at residues #12 and #58 were not essential for starvation-induced degradation and suggested that the lysosomal targeting motifs at these positions were not functional *in vivo*.

Whenever the glutamine at residue #111 was mutated then starvation-induced degradation of RABM was completely inhibited (Fig. 5-7). For wildtype aldolase B sequence in RABM (QQQ), starvation increased degradation of RABMQQQ by greater than twofold, but when glutamine residue #111 was changed to asparagine (QQN) or threonine (QQT and TQT), the resulting mutant RABM failed to show an increase in its degradation in response to starvation. Since the loss of inducible degradation occurred even with a minimum change of glutamine to asparagine (QQN), glutamine #111 is clearly essential for starvation-induced degradation of aldolase B and asparagine cannot function in its place. This is consistent with the function of a motif originally described by J. Fred Dice and indicates that aldolase B is a substrate for receptor-mediated targeting to lysosomes.

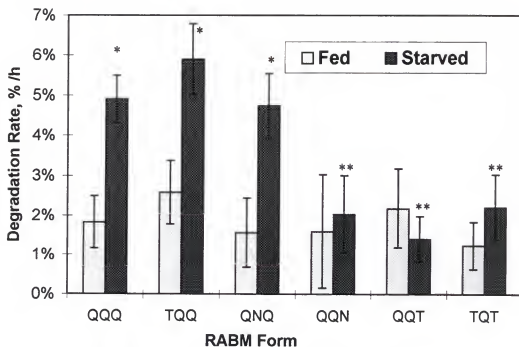


**Figure 5-7: Glutamine Residue #111 is Required for Starvation-Induced Degradation of Aldolase B:** Various forms of RABM were transiently expressed in HuH7 cells, then fed and starved degradation rates  $\pm$  standard error ( $n = 18$  to  $33$ ) for RABM were measured as described in Materials and Methods. Different forms of RABM are labeled according to the mutation code given in Table 5-1. Student's t-test: \*, Starved > Fed,  $p < 0.0001$ ; \*\*, mutant < wildtype = QQQ,  $p < 0.0001$ .

#### Glutamine #111 Specifically Mediates Starvation-Induced Degradation

Above, it was demonstrated that changing glutamine #111 of aldolase B affects its starvation-induced degradation. Here, it is proposed that this change directly affects a degradative mechanism, but if some other function of this enzyme is blocked, then inhibition of starvation-induced degradation might be an indirect affect of other lost function. To address this, evidence that various aldolase functions were similar between

the RABM containing wildtype sequence (QQQ) and mutants at glutamine #111 (QQN, QQT, and TQT) was collected.

Table 5-4 summarizes evidence that mutations at glutamine #111 were specific for blocking the starvation-induced degradation of aldolase B. First, expression levels were similar to wild type RABM, including transfection efficiencies (Table 5-4, column 2) and immunoprecipitated RABM (Fig. 5-3). Second, aldolase is known to bind f-actin (O'Reilly and Clarke, 1993; Wang, et al., 1996), and HuH7 cells have well developed stress fibers that became decorated with antibodies specific for RABM (Fig. 5-8, 9E10). Localization of RABM to actin was confirmed by double labeling with phalloidin which specifically labels f-actin (Figure 5-8b). For all mutant RABM's used in degradation experiments, similar stress fiber morphology (Stress Fiber Label) was observed as with RABM-specific label in RABMQQQ wildtype expressing cells (data not shown). This provided evidence that some actin-binding activity was retained in these mutants (Table 5-4, column 3). Third, aldolase is known to bind GAPDH (Clarke et al., 1982), and when mild conditions were used for immunoprecipitations (Fig. 5-3, Aldolase B) a 42 kD protein was specifically pulled down. Though its identity was not confirmed, the sized matched that predicted for the 42 kD subunits of GAPDH (Clarke et al., 1982). The results suggest that GAPDH binding was retained by site-directed mutants of RABM (Table 5-4, column 4). Above, enzymatic activities were already shown to be comparable to each other regardless of the mutations (Fig. 5-6, Table 5-4, column 5). Lastly, there were no significant differences between various basal degradation rates for all the assayed RABM forms, including the mutants. Furthermore, mutation of

**Table 5-4: Summary of RABM Forms Expressed in HuH7 Cells**

| <b>RABM Form<sup>1</sup></b>                                               | <b>Transient Expression (% Brightly Labeled Cells)</b> | <b>Stress Fiber Label<sup>2</sup></b> | <b>Binds to a 42 kD protein<sup>3</sup></b> | <b>Aldolase Activity<sup>4</sup></b> | <b>Basal Degradation of RABM<sup>5</sup></b> | <b>Starvation-Induced Degradation of RABM<sup>6</sup></b> |
|----------------------------------------------------------------------------|--------------------------------------------------------|---------------------------------------|---------------------------------------------|--------------------------------------|----------------------------------------------|-----------------------------------------------------------|
| <b>QQQ</b>                                                                 | 10-25%                                                 | +                                     | +                                           | +                                    | +                                            | +                                                         |
| <b>TQQ</b>                                                                 | 10-25%                                                 | +                                     | +                                           | +                                    | +                                            | +                                                         |
| <b>QNQ</b>                                                                 | 5-15%                                                  | +                                     | +                                           | +                                    | +                                            | +                                                         |
| <b>QQN</b>                                                                 | 10-25%                                                 | +                                     | +                                           | +                                    | +                                            | —                                                         |
| <b>QQT</b>                                                                 | 10-25%                                                 | +                                     | +                                           | +                                    | +                                            | —                                                         |
| <b>TQT</b>                                                                 | 10-25%                                                 | +                                     | +                                           | +                                    | +                                            | —                                                         |
| Mutants Expressed too Low for Additional Characterization (n/d, not done): |                                                        |                                       |                                             |                                      |                                              |                                                           |
| <b>QTQ</b>                                                                 | <0.1%                                                  | ±                                     | n/d                                         | n/d                                  | n/d                                          | n/d                                                       |
| <b>TTQ</b>                                                                 | <0.1%                                                  | ±                                     | n/d                                         | n/d                                  | n/d                                          | n/d                                                       |
| <b>QTT</b>                                                                 | <0.1%                                                  | ±                                     | n/d                                         | n/d                                  | n/d                                          | n/d                                                       |
| <b>QTN</b>                                                                 | <0.1%                                                  | ±                                     | n/d                                         | n/d                                  | n/d                                          | n/d                                                       |
| <b>TTT</b>                                                                 | <0.1%                                                  | ±                                     | n/d                                         | n/d                                  | n/d                                          | n/d                                                       |

<sup>1</sup> See mutation codes in Table 4-2<sup>2</sup> + : common, often bright; ± : occasional, usually dim, observation based on rare labeling<sup>3</sup> + : specific co-precipitation of an endogenous 42 kD protein (probably GAPDH), see Fig. 4-28<sup>4</sup> + : activity in immunoprecipitate lacking 40 kD protein (endogenous aldolase), see Fig. 4-29<sup>5</sup> + : basal degradation  $\cong$  published values (1.0-2.5%/h); see Fig. 4-31<sup>6</sup> + : starved degradation > basal; — : starved degradation  $\cong$  basal; see Fig. 4-31

glutamine #111 specifically inhibited the induced portion of RABM degradation,

indicating that the basal degradation of mutated RABM's continued during starvation.

The data support that the basal degradation of RABM continued without being affected by the site-directed mutations (Table 5-4, column 6). Together the results demonstrate that glutamine #111 in aldolase B is likely to play a direct role in receptor-mediated targeting to lysosomes induced by starvation.

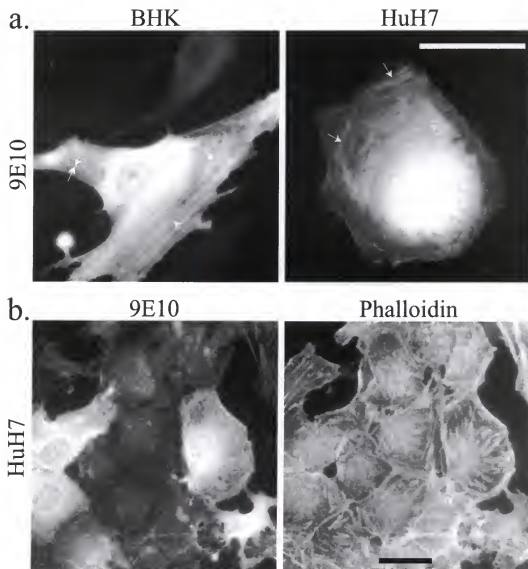


**Figure 5-12: RABM has Actin Binding Activity.** a) left panel, BHK (baby hamster kidney) and right panel, HuH7 cells were transfected for transient RABM expression and single labeled for 9E10 immunofluorescence microscopy as described in Materials and Methods, field showing example of occasional cells having good stress-fiber labeling indicated by arrows; b) HuH7 cells were labeled as in (a) and then double stained with phalloidin carrying an alternative fluorophore, the same field is shown: left panel, the 9E10 fluorescence channel; right panel, the phalloidin fluorescence channel.

At the end of Chapter 3 and the beginning of this chapter, starvation-induced degradation of aldolase B was examined in hepatoma cell lines. It was confirmed that starvation-induced degradation occurs by autophagic degradation for long-lived proteins in general including aldolase B. I found a residue, glutamine #111 that was specifically essential for starvation-induced degradation of aldolase B, indicating that it served as part of a degradative targeting signal. Together, the results of Chapters 3 through 5 support the hypothesis that stress-induced degradation of aldolase B requires ubiquitination and a receptor-mediated targeting signal.

## CHAPTER 6: SUMMARY AND CONCLUSIONS

### Introduction

A model was presented showing two mechanisms known to mediate stress-induced degradation of long-lived proteins (Fig. 1-4). In Figure 6-1, the findings of this study were used to update the initial model with aldolase B utilized as a representative long-lived cytosolic protein. Evidence indicated that autophagy, ubiquitination, cytosolic proteolysis, and a molecular recognition signal function in the degradation of aldolase B in response to heat stress and starvation.

### Autophagy and Ubiquitination

This study began by identifying ubiquitinated forms of aldolase B with antibodies raised against ubiquitin-free aldolase B. In Figure 6-1, ubiquitination is represented by process 6 which maintains aldolase B partially denatured as indicated by preferential reactivity with antibodies to denatured epitopes (Fig. 3-2). Unmodified aldolase B predominated in cytosol, whereas ubiquitinated aldolase B was enriched in lysosomal compartments, especially autophagic vacuoles (Fig. 1-3 and 3-3). Furthermore, lysosomal association of a major ubiquitinated form of aldolase B (Ub68) increased during amino acid starvation and decreased when autophagy was inhibited. Ub68 also occurred in E36 cells that undergo increased autophagic degradation during heat stress

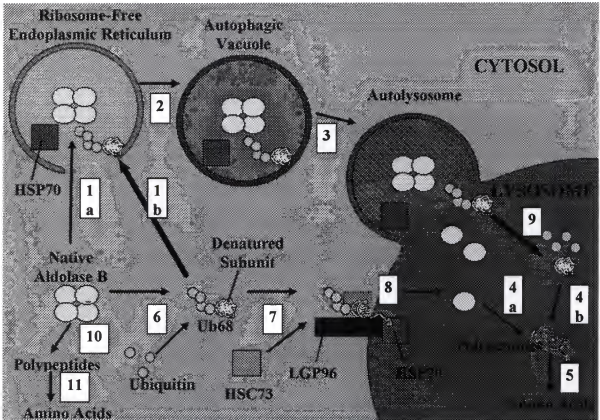


**Figure 6-1: Mechanisms for Stress-Induced Degradation of Cytosolic Proteins in Lysosomes.** Autophagy (upper pathway) and receptor-mediated targeting (lower pathway) were proposed for stress-induced delivery of cytosolic proteins to lysosomes for degradation; the arbitrary cytosolic protein is shown as a tetramer (aldolase B occurs as a tetramer); components of the pathways are labeled on the diagram; processes are labeled by boxed numbers: 1a & b, association with or engulfment by pre-autophagic membranes; 2, sequestration into double-membrane bound autophagic vacuole; 3, maturation of autophagic vacuole (acidification and acquisition of lysosomal hydrolases); 4a & b, proteolysis into polypeptide fragments; 5, complete degradation to amino acids; 6, disassembly and denaturation of structure by an unknown factor; 7, association with a receptor complex on the lysosomal surface; 8, translocation across the lysosomal membrane; 9, limited proteolysis and removal of ubiquitin; 10, proteolysis into polypeptide fragments; 11, complete degradation to amino acids.

(Fig. 3-11). In response to heat stress, endogenous aldolase A of E36 cells accumulated in lysosomes dependent upon elevated levels of ubiquitination (Fig. 3-5). These results

suggested that ubiquitination facilitated the autophagic delivery of aldolase B to lysosomes (Fig. 6-1, process 1b > 1a), but other data indicate that autophagic degradation is affected at a later step in the pathway.

Ubiquitination was previously proposed to mediate degradation in autolysosomes (Fig. 6-1, process 4) rather than sequestration, because autolysosomes expanded sixfold in volume when ubiquitination was inhibited (Lenk et al., 1992). Consistent with this, lysosomal degradation was temperature-dependent (Fig. 6-12), increased approximately 6-fold during heat stress relative to control temperature (Fig. 6-13), and required ubiquitination (Figs. 3-14 and 3-15). Additionally, ubiquitination was required for limited proteolytic processing of an epitope-tagged form of aldolase B associated with lysosomes (Fig. 3-10). This evidence supports a role for ubiquitination in proteolysis mediated by at least some lysosomal proteases (Fig. 6-1, process 9).

Whether for delivery or degradation, this study demonstrates that ubiquitination mediates lysosomal mechanisms for degradation of aldolase B. The hypothesis that aldolase B requires ubiquitination for enhanced degradation in lysosomes was supported. Given this, a number of questions arise. What must be ubiquitinated, aldolase B or a component of the autophagic mechanism? Is ubiquitinated aldolase B sequestered or degraded faster than unmodified aldolase B? What does ubiquitination do to the structure and function of aldolase B?

Preliminary evidence indicates that ubiquitinated aldolase B is maintained in a more denatured conformation. Does ubiquitination mediate disassembly of aldolase

tetramers? Does it expose potential targeting signals for degradative mechanisms? Is ubiquitinated aldolase B preferentially degraded? Ubiquitinated aldolase B constitutes at least part of Ub68, one the most abundant protein-ubiquitin conjugate species characterized on SDS-PAGE. Though enriched in lysosomes relative to total proteins, a significant amount of Ub68 still occurs in the cytosol (Fig. 1-3 and 3-3). Does aldolase B make up most or all of Ub68? How abundant is Ub68 relative to unmodified aldolase B? Is Ub68 a necessary intermediate for ubiquitin-mediated degradation of aldolase B? Could ubiquitinated aldolase B perform another function?

#### Clues from Temperature-Dependent Cytosolic Proteolysis and Lysosomal Degradation

While examining heat stress-induced autophagic degradation, a second mechanism for aldolase B degradation during heat stress was identified. The degradative activity was resistant to treatments that inhibited autophagy and lysosomal degradation, suggesting a cytosolic mechanism (Fig. 6-1, processes 10 and 11). Temperature-dependency of this cytosolic mechanism was consistent with thermal enhancement of a rate-limiting reaction. Calculations of the activation energy ( $E_a$ ) were limited by use of only three temperatures (Fig. 4-12), but the large magnitude was consistent with regulated degradation by the complete 26S proteasome complex. An initial  $E_a$  estimate was 28 kcal/mole (data not shown), but when more data were included  $E_a$  was 35-40 kcal/mole. This was higher than predicted for proteolysis by isolated 20S proteasomes (27 kcal/mole), supporting the concept that a rate limiting step for proteasomes occurs before 20S-mediated proteolysis by 19S regulatory components of the entire 26S

proteasome complex (Coux, et al., 1996). However,  $E_a$ 's estimated for cytosolic proteolysis of aldolase B could not distinguish between  $E_a$ 's predicted for autophagic degradation (35 kcal/mole) and regulated proteolysis in 26S cytosolic proteasomes (presumably >27 kcal/mole). The inhibitors used here measurably blocked lysosomal degradation of total TCA proteins, indicating that a cytosolic protease, consistent with 26S proteasomes, mediated proteolysis of aldolase B.

Stress-induced degradation in proteasomes is known to be regulated by ubiquitination, but when ubiquitination was inhibited, cytosolic proteolysis of aldolase B continued. However, inhibition of ubiquitination was incomplete (Gropper, et al., 1991) and could not eliminate the possibility that cytosolic proteolysis continued at low levels of ubiquitination. Consistent with this, similar amounts of Ub68 (ubiquitinated aldolase B) occurred in cells having a predicted fourfold difference in ubiquitination activity (Fig. 3-11).

During heat stress, lysosomal inhibition did not affect enhanced cytosolic proteolysis of aldolase B (Figs. 4-4 and 4-11) but effectively blocked enhanced degradation of total long-lived TCA-precipitable peptides (Fig. 3-15). If aldolase B is representative of long-lived housekeeping proteins, then enhanced cytosolic proteolysis of aldolase B (loss of immunoreactivity) was limited to production of undetected TCA precipitable polypeptides, and enhanced degradation of total proteins (loss of TCA precipitability) proceeded to amino acids in lysosomes via autophagy. The results are consistent with a role for proteasomes in aldolase B degradation, because cytosolic

proteasomes produce small polypeptides rather than amino acids (Lowe et al., 1995; Coux, et al., 1996), whereas lysosomes completely degrade proteins to amino acids (Hershko and Ciechanover, 1982; Mortimore and Poso, 1987; Olson et al., 1990).

Autophagic degradation of TCA precipitated proteins and partial cytosolic proteolysis of aldolase B correlated with temperature such that their rates correlated with each other (Fig. 4-12). This suggested that both mechanisms could play a role in heat stress-induced degradation of aldolase B by thermodynamic stimulation of molecular machinery. Cytosolic partial proteolysis was faster than autophagic degradation under similar conditions (Fig. 4-13), suggesting the possibility that a product of the former might stimulate the latter. Are lysosomes regulated by products of proteolysis? Amino acids (lysosomal products) have been established to inhibit autophagic degradation. Is it possible that peptides produced by cytosolic proteases like the proteasome have an opposite effect, stimulating autophagy?

Endogenous inhibitors of the proteasome have been proposed to prevent excessive proteolysis of cellular proteins (Coux, et al., 1996). One of the best characterized endogenous inhibitors, CF-2, was found to be identical to  $\delta$ -aminolevulinic acid dehydratase (AADH), an essential enzyme in heme biogenesis (Guo, et al, 1994). AADH is a long-lived oligomeric housekeeping protein with 40 kD subunits that occur as a 50 kD ubiquitinated form associated with 26S proteasomes (Guo, et al, 1994). Above, aldolase B was described as a long-lived oligomeric housekeeping protein with 40 kD subunits that occur as a 68 kD ubiquitinated form. The parallel suggests that

aldolase B might be another endogenous proteasome inhibitor. Consistent with this, the proteasome has a stable intermediate during carboxyl to amino terminal proteolysis of substrates (Lowe et al., 1995), and aldolase B has a protease-sensitive carboxyl terminus (Chapter 1). The proteasome might begin proteolysis on the accessible carboxyl terminus but be blocked by the protease-resistant structure for the rest of aldolase B. Stress-induced ubiquitination of aldolase B could relax its structure, allow it to degrade, and thereby make the proteasome available to degrade other proteins. Though the identity of the cytosolic protease involved in the degradation of aldolase B still needs to be established, a role for the proteasome seems important to examine.

#### Signal-Mediated Targeting

Aldolase was found to have properties similar to substrates of receptor-mediated targeting to lysosomes (Fig. 6-1, processes 7 and 8), including three potential targeting signals in the aldolase B sequence (Fig. 1-2). Site-directed mutagenesis was used to replace glutamines defined as "essential" for each of these motifs. Of these, only glutamine #111 was shown to specifically mediated starvation-induced degradation of aldolase B. Whether aldolase B utilizes the same mechanism as previously described for receptor-mediated targeting to lysosomes was not determined, but the data are consistent with this mechanism. Previously, receptor function for this pathway was demonstrated in cultured cells (Cuervo, et al., 1996), but signal function had not. If glutamine #111 mediates this mechanism for aldolase B, then this study provides the first evidence for signal function in living cells.

A major weakness of receptor-mediated targeting to lysosomes was recently published (Gorinsky, et al., 1996). Conformations of known substrates for this pathway would prevent receptor recognition, prompting investigators to propose unknown factors to create more extended conformations (Gorinsky, et al., 1996). Based on native and denatured immunoreactivities, ubiquitinated aldolase B retained a more denatured conformation than unmodified aldolase B. This suggested that ubiquitin can function as the unknown factor of Figure 1-1 to relax the structure of aldolase B and improve lysosomal targeting signal availability (Fig. 6-1, process 6 and 7).

While characterizing the signal-mediated degradation of aldolase B, rat and human aldolase B were expressed in rat and human cells. Maximal degradation rates occurred when the source animal species of the aldolase B matched the species of the cell (Fig. 5-5, RAB in Fao and HAHAB in HuH7). When species did not match (HAHAB in Fao and RABM in HuH7), starvation-induced degradation rates were 35-40% lower (Student's *t*-test,  $p < 0.001$ ). This indicated that species specific differences between rat and human aldolase B might mediate recognition by proteolytic machinery. Between rat and human aldolase B, there are seventeen non-identical amino acid residues; only twelve alter side chain chemistry (Fig. 1-2). These residues are not near the primary amino acid sequence for the signal at glutamine #111, indicating that some might function as an independent recognition signal for cellular degradative mechanisms. Though more work is needed to confirm these initial findings, the ability of cells to distinguish between rat and human isoforms would further support signal-mediated

targeting of aldolase B during starvation. This study supports the hypothesis that a molecular signal mediates stress-induced mechanisms for degradation of aldolase B.

#### Present and Future Contributions to the Field of Protein Turnover

This study demonstrates that Aldolase B is a substrate for multiubiquitination and ubiquitin-mediated proteolysis in lysosomes. This is consistent with the hypothesis that ubiquitination of substrate proteins facilitates degradation in lysosomes (Lenk, et al., 1992). A secondary hypothesis is that ubiquitination is required for a subpopulation of lysosomal hydrolases. In support of this, limited proteolysis of aldolase B in lysosomes was blocked when ubiquitination was inhibited (Fig. 3-10), and aldolase B did not significantly accumulate in lysosomes, indicating other proteolytic activities continued. In the future, the recombinant aldolase B (RABM) can be used as a marker for ubiquitin-dependent limited proteolysis in lysosomes. As such, limited proteolysis of RABM could serve as an assay for this activity, allowing purification and identification of specific lysosomal components that require ubiquitination.

When aldolase A was examined, heat stress cause greater than twofold accumulation of aldolase enzyme activity in lysosomes, and this accumulation was ubiquitin-dependent (Fig. 3-5). This indicated that ubiquitination plays a role in sequestration of aldolase A. In support of this interpretation, ubiquitinated forms of aldolase B (Ub68) were enriched more in autophagic vacuoles than in cytosol or lysosomes (Fig. 1-3a). Together, the data suggest that ubiquitination mediates at least two processes during lysosomal degradation: sequestration and lysosomal proteolysis.

Autophagic sequestration in starved mammalian cells was proposed to occur by bulk non-selective uptake (Kopitz et al., 1990). However, in starved liver cells, ubiquitinated aldolase B was enriched in lysosomes, while unmodified aldolase B was enriched in cytosol (Fig. 3-3). Assuming ubiquitination occurs in cytosol, the results contradict a non-selective mechanism, and suggest that ubiquitinated proteins can undergo selective sequestration. Selective autophagy occurs in yeast (Tuttle et al., 1993). Aldolase B might provide valuable evidence for ubiquitin-mediated selective autophagy. The different SDS-PAGE mobility of 40 kD unmodified and 68 kD ubiquitinated (Ub68) aldolase B can be used to separate them after co-immunoprecipitation (Fig. 3-11). In pulse-chase experiments, the relative loss of radiolabel from these two forms could be compared with and without autophagic inhibition. The results would determine relative degradation rates for aldolase B and Ub68 and their relationship with autophagic mechanisms. If ubiquitination of aldolase B makes it a better substrate for autophagy, then autophagic degradation of Ub68 would be more rapid than for 40 kD aldolase B.

Amino acids have long been known to inhibit autophagic degradation, but how their levels are detected and translated into an inhibitory signal is unknown. Temperature-dependence for cytosolic proteolysis of aldolase B and lysosomal degradation of long-lived proteins were parallel with statistically equal activation energies (Fig. 4-12). Equal activation energies are predicted for processes controlled by the same rate limiting mechanism. If cytosolic proteolysis of long-lived proteins is partial

as proposed for aldolase B above, then cytosolic peptides would be produced. If these peptides induce autophagy, then autophagic degradation would be dependent on the rate-limiting step for cytosolic proteolysis and give the same  $E_a$ . This suggests a model in which amino acid deprivation induces limited proteolysis which produces peptides which induce autophagy. Autophagic degradation in lysosomes produces amino acids that might inhibit cytosolic proteases that produce peptides, causing peptide levels and peptide-dependent autophagy to be reduced. Such a model could be tested by introducing peptides into cytosol with liposomes, electroporation, microinjection or overexpression from plasmid vectors then measuring autophagic degradation. If the model is correct, then peptides should induce autophagy even in the presence of inhibitory amino acids.

As discussed previously, the proteasome is the major protease in cytosol, is known to produce peptides, and might mediate cytosolic proteolysis of aldolase B. To examine this, chemical inhibitors of proteasomes can be tested for their ability to block cytosolic proteolysis of aldolase B. This will have to be done in the presence of lysosomal inhibition to detect the cytosolic protease activity while excluding lysosomal degradative mechanisms. It was also proposed that aldolase B might be an endogenous inhibitor of proteasomes. Proteasomes have been effectively purified from cells, and *in vitro* inhibition of its activity aldolase B can be tested. This study purified catalytically active recombinant aldolase B expressed in *E. coli*. The protease-sensitive carboxyl terminus of aldolase B would be a reasonable site for initiation of proteolysis by the

proteasome. Carboxyl-terminally truncated recombinant aldolase B could be purified to determine if the “loose” carboxyl terminus is necessary for interaction with the proteasome. In this way, it might be shown that aldolase B is a new endogenous inhibitor for the proteasome or a mechanism for protein interaction with the proteasome might be established.

The last major find in this study is the existence of a targeting signal in aldolase B required for starvation-induced degradation. Whether aldolase follows the currently proposed mechanism for receptor-mediated pathway is not known. This could be established by *in vitro* assays developed by J. Fred Dice using known substrates to compete for the pathway. Transient overexpression of the receptor for this pathway, LGP96, in cultured cells was shown to more than double long-lived protein degradation (Cuervo et al., 1996). In this study, transiently expressed aldolase B had degradation rates similar to those for endogenously expressed aldolase B. By coexpressing LGP96 and aldolase B, degradation of aldolase B should be increased, and this increase should not occur for aldolase B with a mutated targeting signal (altered glutamine #111). This would demonstrate that aldolase B is a bonafide substrate for the established pathway and would be the first demonstration of signal function in living cells. If aldolase B degradation is not mediated by LGP96, then an alternative recognition mechanism or receptor would be indicated for aldolase B. If so, future effort would focus on characterizing the nature of the signal in aldolase B and identifying the novel recognition molecules mediating its starvation-induce degradation.

In conclusion, ubiquitinated forms of aldolase B (*e.g.* Ub68) associated with autophagic vacuoles and lysosomes during nutrient stress (starvation) in rat liver and the Fao rat hepatoma cell line. Ubiquitinated aldolase B that maintained a denatured conformation possibly exposing recognition signals was detected in rat liver, Fao cells, and in the E36 Chinese hamster lung cell line. During heat stress, accumulation of endogenous aldolase A in lysosomes and lysosomal proteolytic processing of exogenous aldolase B required ubiquitination in E36 cells. Heat stress caused ubiquitin-mediated autophagic degradation of long-lived proteins in E36 cells. Together the results support a role for ubiquitination in the stress-induced degradation of aldolase B. Basal and starvation-induced degradation of transiently overexpressed aldolase B was like that for endogenous aldolase B, in the HuH7 human hepatoma cell line. A mutated (one amino acid changed) lysosomal targeting signal in aldolase B specifically prevented starvation-induced degradation of the mutant protein with no effect on other aldolase B properties. Together, these results supported the original hypothesis that during stress, aldolase B requires both ubiquitination and a receptor-mediated targeting signal for enhanced degradation in lysosomes.

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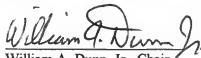
## BIOGRAPHICAL SKETCH

In 1986, Peter P. Susan earned his Bachelor of Science degree from the Pennsylvania State University at State College, Pennsylvania. Early in his studies he majored in computer science then changed his major to microbiology. When finishing his undergraduate work, Mr. Susan moved to Gainesville, Florida where he enrolled in the Vertebrate Zoology Program of the College of Liberal Arts and Sciences at the University of Florida and worked full-time as a laboratory technician in the Departments of Zoology, Botany, and Biological Sciences, providing support for research and teaching. During this work, he prepared technical illustrations published in laboratory manuals for the University of Florida.

In 1988, Peter Susan then successfully completed a Masters Fellowship for Teachers in the Department of Instruction and Curriculum of the College of Education at the University of Florida, earning a master of education degree in science instruction and curriculum. For several years, he served as a public school science teacher in Manatee County, Florida.

By spring of 1992, Mr. Susan pursued and acquired a research assistantship in the Department of Anatomy and Cell Biology of the College of Medicine at the University of Florida. In 1993, he began studying protein turnover in the laboratory of Dr. William A. Dunn, Jr. This dissertation resulted from much of that work.

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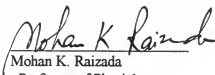
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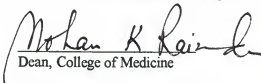
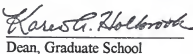
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